SOMACLONAL VARIATION AND INDUCED MUTATIONS IN CROP IMPROVEMENT

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Somaclonal Variation and Induced Mutations in Crop Improvement

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Preface

Genetic variability is an important parameter for plant breeders in any conventional crop improvement programme. Very often the desired variation is unavailable in the right combination, or simply does not exist at all. However, plant breeders have successfully recombined the desired genes from cultivated crop germplasm and related wild species by sexual hybridization, and have been able to develop new cultivars with desirable agronomic traits, such as high yield, disease, pest, and drought resistance. So far, conventional breeding methods have managed to feed the world's ever-growing population. Continued population growth, no further scope of expanding arable land, soil degradation, environmental pollution and global warming are causes of concern to plant biologists and planners. Plant breeders are under continuous pressure to improve and develop new cultivars for sustainable food production. However, it takes several years to develop a new cultivar. Therefore, they have to look for new technologies, which could be combined with conventional methods to create more genetic variability, and reduce the time in developing new cultivars, with early-maturity, and improved yield.

The first report on induced mutation of a gene by H.J. Muller in 1927 was a major milestone in enhancing variation, and also indicated the potential applications of mutagenesis in plant improvement. Radiation sources, such as X-rays, gamma rays and fast neutrons, and chemical mutagens (e.g., ethyl methane sulphonate) have been widely used to induce mutations. Phil Larkin, in the 'Introduction' in this book, has indicated that the 'the role of induced mutations in plant breeding has been a controversial subject for several decades'. Now it is a well-established fact that mutation techniques have made a significant contribution in the improvement of crops, vegetables, woody plants, ornamentals, medicinal plants and herbs. However, mutation breeding has not been common in forest trees. Mutant cultivars have been released in more than 50 countries. The top six countries are China, India, the former Soviet Union, The Netherlands, Japan and the USA. The FAO/IAEA Mutant Varieties Database indicates that during the past decade more than half of the induced mutants were released as cultivars. So far more than 1700 mutant cultivars, involving 154 plants, have officially been released. The important traits, such as plant height (dwarf), plant habit, photoperiod response (day-length insensitivity), flower and fruit colour, flower and fruit shape, fruit flesh colour, seedlessness, non-seed-shattering, tuber size, male sterility, earliness, high yield, high protein and oil, disease and/or insect resistance, and tolerance to environmental stresses, were the main features of mutant cultivars.

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The alternative approach to the use of chemical and physical mutagens is insertional mutagenesis, and this is becoming an invaluable tool in molecular biology for plant improvement. Insertional mutagenesis allows a connection between an observed phenotype and a gene. This has been achieved with gene tagging with transposons and T-DNA insertional mutagenesis. The *Agrobacterium*-mediated gene transfer system is widely applied for insertional mutations, and requires the production of transgenic plants, and screening large numbers of individuals for any change in the structure or function of interest.

Tissue culture derived plants show variation termed somaclonal variation. This phenomenon has been controversial from the very beginning. The study of the type of variation, frequency, spectrum and transmission of variation thus produced is still limited to a few plants, especially when it comes to the use of such variations in plant breeding. This is perhaps due to the fact that such variations are unpredictable in nature and can either be heritable (genetic) or non-heritable (epigenetic), and not used perhaps due to a lack of interaction of tissue culturists with plant breeders. *In-vitro* culture, in combination with mutation techniques, offers several advantages to overcome some of the problems of conventional breeding, e.g. mutagenic treatment of large populations of cells, somatic embryos, apical and axillary buds, micro-sized plants, and multiplication of selected genotypes in a small space. The success of applying somaclonal variation in plant breeding is very much dependent on the genetic stability of the selected somaclones and a close interaction with plant breeders.

The basic purpose of this book is to introduce readers to the relative value of each method in the improvement of various agricultural crops by using parasexual techniques. While no book can be a complete source of all information on a subject, a range of topics were selected to highlight the recent developments in mutagenesis (conventional approach and molecular biology) and *in-vitro* induced variation in plant breeding. We have deliberately kept the title of this book short, to emphasize somaclonal variation and induced mutations. The book is divided into three sections. Section 1 contains 13 chapters, mainly on somaclonal variation (SCV) in crop improvement, ornamentals, cereals, forage grasses, banana, and forest trees, cytogenetic basis of SCV, *in-vitro* selection, gametoclonal variation, protoclonal variation, and Solanaceous medicinal plants. Section 2 deals with induced mutations, and is covered in 9 chapters, mainly on mutagenesis in sugarcane, fruit trees, apomixis, ornamental plants, disease resistance, cereals, legumes, and vegetatively propagated plants. Section 3 describes the molecular aspects of mutagenesis, somaclonal variation, and insertional mutagenesis (T-DNA mutagenesis, transposons), molecular methods for identifying somaclonal variation, RAPD markers in banana somaclones, and transgene expression.

All manuscripts were reviewed by two persons, and revised accordingly. We were overwhelmed by the response of research scientists who gave freely their time to review the manuscripts. The reviewers were: Drs B.S. Ahloowalia, D.S. Brar, S.M. Jain, Teemu Teeri, A. Shulman, Marc Von Montagu, J.M. Bonga, K.P. Pauls, I.K. Vasil, J. Janick, R.E. Veilleux, G.J. De Klerk, A. Ashri, P.K. Gupta,

A. Cassells, Dan Lineberger, Steven Gelvin, J. Preece, F.J. Hammerschlag, P. Larkins, M.R. Ahuja, K. Ishii, S. Baezinger, W. Preil, D.R. Vuylsteke, E. Amano, M.M. Fitch, J.J.M.R. Jacobs and Katia Libre.

We hope that somaclonal variation and induced mutagenesis, together with plant breeding, will become a desirable approach in plant improvement. The application of molecular biology tools should become more prominent, to discover the causes of somaclonal variation, induced mutations, and finally in gene identification and transformation. In woody plants molecular markers will be of great advantage to identify genetic variability at early stages of development, and for use as a 'diagnostic kit'. We sincerely hope that our efforts in compiling this book will be of some value in stimulating the thoughts of researchers and students for future plant breeding activities in crop improvement.

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I express my thanks to Dr K. Ishii for his hospitality during my visit as a senior STA Fellow in his laboratory, Tsukuba, Japan. My Japanese colleagues, I. Kinoshita and R. Thakur, were helpful in using computer facilities. The timely help of Professor E. Pehu and Tapio Poutala of our Plant Production Department is very much appreciated.

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S.M. Jain
Book Project Leader

SECTION 1

1. Introduction

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The first report on induced mutation of a gene indicated the potential application of mutagenesis for plant improvement (Muller, 1927). Five decades later Brock (1980) was able to write 'The role of induced mutations in plant breeding has been a controversial subject for several decades.'

It is now well established that mutation breeding has made a significant contribution to plant improvement. While mutations which arise in plant cell and tissue culture, termed *somaclonal variation*, may have a different basis of origin or a different spectrum of types, they are rightly considered together with mutations induced with physical and chemical mutagens.

Induced Mutations for Crop Improvement

Induced mutations have been directly useful in crop improvement, and this is the emphasis of this book. The chapters of the first half of the book will substantiate the positive early indication regarding tissue culture-induced mutation. The second half will also give ample testimony that this is true for chemical- and radiation-induced mutation. More than 1700 mutant varieties involving 154 plant species have been officially released (Maluszynski *et al.*, 1995). I offer one recent example which demonstrates the development of an entirely new crop by mutation. The non-edible oil from linseed flax, *Linum usitatissimum*, has been turned into a new oilseed crop (linola) and a new industry of great potential. This was achieved by the combination of two induced mutations of the fatty acid synthesis pathway resulting in greatly reduced linoleic acid content (Green, 1986; Green and Dribnenki, 1994). The oil which formally would go rancid, and therefore had only industrial uses, was now comparable to sunflower and canola oils for food uses.

Micke *et al.* (1987) assembled an impressive amount of information as to the usefulness of induced mutations in plant improvement. This includes 539 released new cultivars of seed-propagated species and 305 released and new varieties of vegetatively propagated species. Over 90% of these were developed from radiation. Over 1000 new mutant varieties have been produced and released in the past 15 years. These authors make the point that there is no longer a need to prove that a mutation has been caused by the mutagenic agent, and therefore it would be more efficient to mutagenize heterozygotes. This not only exposes more alleles to the mutagenesis, but also opens new possibilities for novel genotypes resulting from somatic recombination.

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The Value of Mutations to Basic Biochemistry and Genetics

Mutations, induced by any method, have been as valuable for theoretical genetic studies as they have for crop improvement. Mutations have created markers, or genetic signposts, to enable the mapping of various species. More recently, the majority of markers in saturated genetic maps tend to be random and undefined sequences, detected with molecular techniques (notably randomly amplified polymorphic DNA and restriction fragment length polymorphism). In addition, mutants of specific steps in biochemical pathways have been of great utility in determining the chemical steps and their order in several major biochemical pathways.

Mutation by insertion has become a particularly valuable tool in molecular biology. This could prove the greatest contribution of mutational research to plant improvement in the future. Insertional mutations serve as stepping stones in the cloning of genes which control important plant processes, and thereby they permit the genetic manipulation of those processes to optimize their usefulness in the production of food, fibre and animal feed. Insertional mutations allow a connection to be made between an observed phenotype and a gene. This so-called *gene tagging* has been achieved with transposons, sequences which are able to physically alter their position in the chromosomes. It has also been achieved by the primary transformation events such as the *Agrobacterium*-mediated T-DNA insertion. Especially in the latter case, the researcher is required to produce transgenic plants and to screen large numbers of individuals for an alteration in the structure or function of interest. The change is presumed to be caused by the insertional disruption of a gene controlling that function or structure. In both cases, the researcher has a molecular probe (the transposon or the T-DNA sequence) which allows the cloning of genes in proximity to the inserted sequence. For example, using a transposon tag, the L6 gene conferring flax rust resistance has recently been cloned (Lawrence *et al.*, 1995). The phenotype that was assayed for was the creation of a susceptible from a resistant background.

The Controversy over Somaclonal Variation

It would be fair to say that the phenomenon of *somaclonal variation* has been controversial from the beginning. Of course observations of variant plants regenerating from cell and tissue cultures preceded the coining of the terms *phenovariants* (Sibi, 1976), *calliclonal* (Skirvin and Janick, 1976), *protoclonal* (Shepard, 1981), *somaclonal* (Larkin and Scowcroft, 1981) or *gametoclonal* variation (Evans *et al.*, 1984). The controversy at first focused on whether the variation existed at all. Some argued that the genetic differences claimed were due to poorly designed experiments, stray pollen or poor controls. Few now doubt that somaclonal variation occurs. Metakovsky *et al.* (1987) argued that the variation we reported (Larkin *et al.*, 1984) in the patterns of wheat storage proteins in first or second generation grain of regenerated plants was the result of heterozygous

material going into culture or stray pollen after culture. More recently, the same authors have published their own evidence that somaclonal variation does in fact occur for wheat gliadins (Upelniek *et al.*, 1995). Our own early work with wheat was in fact done with rigorous controls and painstaking pedigrees of every source plant and every plant regenerated from culture (Larkin *et al.*, 1984). The rigour of the controls was because we anticipated the scepticism, and indeed, carried the burden of our own early doubts.

The controversy shifted to whether somaclonal variation could be useful. The debate in this aspect continues. Indeed, I have had something of a journey on this issue myself. This book will surely make a major contribution to that debate. The authors of the chapters in the second half of the book will themselves be divided on this question, and some will be awaiting further data.

Is Somaclonal Variation Different from Chemical- or Radiation-induced Mutation?

It is still not possible to answer this question with confidence. At the superficial level, there often appears to be a similarity of scope of traits affected. In the cereals the most commonly observed mutations from both methods are those affecting height, awns, head morphology and maturity (heading date). However, this should have been expected, because these are the characteristics of the plants that are most readily observed. In other words, it is a reflection of the common and casual method of screening rather than evidence of a common mechanism or basis.

When screening for mutations becomes more specific and analytical, different outcomes from the two approaches can readily emerge. Sree Ramulu (1982) supplies an excellent example of this. He studied the S-alleles which govern the gametophytic incompatibility in *Lycopersicon*. Extensive and varied induced mutagenesis had failed ever to produce a new S-allele. However, a very small sample of somaclones (from cultured anthers of *L. peruvianum*) revealed a number of new S-specificities in both genotypes in the study. Those new specificities were stable and simply inherited.

Another interesting and accessible point of comparison is the relative frequency of dominant mutation. Brock (1979) analysed decades of induced mutagenic studies and estimated that dominant mutants occur at a frequency of 1% of recessives. My scanning of the somaclonal literature suggests the frequency of dominant somaclonal mutations might be closer to 10%.

Somaclonal mutation also appears to be distinguishable by the putative occurrence of homozygous mutants. The examples continue to grow of non-segregating variants arising directly in the primary regenerants (Sibi, 1976; Evans and Sharpe, 1983; Sun *et al.*, 1983; George and Rao, 1983; Larkin *et al.*, 1984; Gavazzi *et al.*, 1987; Kaeppler and Phillips, 1993). Mitotic recombination has been proposed as a possible mechanism of homozygous somaclonal variation. However, a recent study by Xie *et al.* (1995) failed to demonstrate mitotic recombination at two test

loci. Another hypothesis to explain this phenomenon is that culture often involves cell lineages which become haploid (or monosomic) for a few cell generations. If the mutation occurs on a monosomic chromosome which subsequently doubles, it will be in the homozygous condition. Giorgetti *et al.* (1995) present evidence for the fascinating possibility that cultured plant cells can undergo a form of somatic meiosis to produce haploid cells.

Where Does Somaclonal Variation Come From?

In a previous report, the types of genetic changes associated with culture-induced mutation have been reviewed (Larkin, 1987). These include point mutations, changes to methylation patterns, altered sequence copy number, transposable element movements and chromosomal rearrangements. It is difficult to conceive a single underlying basis for such disparate genetic consequences. Phillips *et al.* (1994) recently proposed a connection to repeat-induced point mutation (RIP) in *Neurospora*. According to the RIP hypothesis, newly C-methylated sequences are prone to deamination (to form thymine) which leads to point mutations. The initial C→T transitions can be mismatch repaired with a non-methylated cytosine; or it can be mismatch repaired to give a G→A point mutation. This new methylated A might retain or lose its methylation in subsequent replication. This is particularly interesting in the light of our recent demonstration that gene promoters can be far more active in plant cells with A methylation than without (Graham and Larkin, 1995). It is well known that C methylation can lead to suppression of gene activity. Therefore, the initial change in C methylation can have waves of consequences through subsequent replication, including point mutations and alterations in promoter activity.

At a higher level, much of somaclonal variation probably arises by genetic recombination and chromosomal rearrangement of one sort or another. In other words, it is not so much a phenomenon of creating variation but rather of uncovering variation. We might argue that there is a genetic resource within the plant. Chromosomal rearrangement might bring about new juxtapositions of genes and controlling sequences, thereby silencing previously active genes and activating others. The recent domesticated history of the major crops and their breeding is likely to have already exploited this variation to a considerable extent. Or perhaps a long history of agronomic selection has already come close to optimizing what we may call this *internal* genotype. If this is true, we might predict that somaclonal variation will frequently be deleterious in the highly bred species. An informative recent study in rice by Mezencev *et al.* (1995) demonstrates the preponderance of deleterious changes but nevertheless the occurrence of some favourable ones. Other rare and favourable somaclonal mutants in major crops include: the *Piricularia* resistant rice cultivar Dama (Hezsky and Simon-Kiss, 1992); drought-tolerant rice (Adkins *et al.*, 1995); glyphosate-tolerant maize (Racchi *et al.*, 1995).

But other species have a shorter history of conventional breeding, are vegetatively propagated or for other reasons might be considered unsophisticated in breeding terms. Favourable variation has been easier to find in such species, for example: yam, *Dioscorea floribunda*, with increased diosgenin content (Sen *et al.*, 1991); Japanese mint, *Mentha arvensis*, with increased herb and oil yield (Kukreja *et al.*, 1991); Indian mustard, *Brassica juncea*, with low glucosinolates (Palmer *et al.*, 1988); fall armyworm resistance in bermudagrass, *Cyanodon dactylon* (Croughan *et al.*, 1994); heat- and drought-tolerant dallisgrass, *Paspalum dilitatum* (Tischler *et al.*, 1993); and a new high-yielding, shattering-resistant Indian mustard cultivar (Katiyar and Chopra, 1995).

It seems likely that the resource of genetic variation exploited by cell culture is available and accessible by other means. Simply the process of intercrossing genetically diverse types may unleash more variation than is evident in the parents themselves. One advantage of writing an Introduction is that I am able to make such a sweeping statement without the burden of proof and simply to be provocative. Episodes of *genomic shock* have been known to occur by other means such as radiation, environmental extremes or interspecific crossing. It appears that cell culture is a particularly effective means to induce such variation.

Breeders have not extensively exploited somaclonal variation. There are varied reasons for this. One is that there has been little time to assemble well-characterized demonstrations of utility that would be necessary to persuade those associated with plant improvement to invest the time and energy into it. For many too, there is a technological barrier which means they have not had the resources to attempt this. In an environment of limited resources, most breeders prefer to invest, in more established methods. Indeed I have counselled many away from somaclonal variation when the problem before them is one which has obvious solutions by more established methods. In wheat, it makes no sense to search for aluminium tolerance by culture-induced variation, with or without *in-vitro* selection, when excellent tolerance genes with single dominant effects are already available in the wheat germplasm banks. By contrast, a better case can be made in lucerne (alfalfa) to look for somaclonally induced tolerance because it is very sensitive to aluminium in acid soils, and sources of tolerance appear not to be available by other means.

Examples of Released Somaclonal Cultivars

Despite the relatively short time since their first description, somaclonally derived mutants are finding their way to the market and to agriculture. The company FreshWorld Farms has been marketing a tomato with altered colour, taste, texture and shelf life since 1993. American Cyanimid is expected to release an imidazolinone herbicide-resistant maize in the next couple of years. There is newly released germplasm of bermudagrass, *Cyanodon dactylon*, called Brazos-R3, with increased resistance to fall armyworm compared to its donor genotype Brazos (Croughan *et al.*, 1994).

The recent example that has most caught my imagination is that of the *Lathyrus sativus* somaclone which no longer accumulates neurotoxin in the grain (Mehta *et al.*, 1994; Yadav and Mehta, 1995). This species is wonderfully adapted to harsh and dry environments in India and can become a crucial food source to desperate farmers in bad seasons. New somaclonal variant lines are now available which have negligible levels (0.03%) of these harmful toxins compared to the parental seed (0.3%). In addition, the new cultivar has increased seed yield and is earlier maturing.

The accumulating examples of useful somaclonal variants and new cultivars derived from them give testimony and some confidence that this approach will make an ongoing contribution to plant improvement. In a few cases, a methodological comparison was set up in which somaclonal variation delivered the desired mutants while chemical or gamma treatments did not (e.g. Gavazzi *et al.*, 1987). On the other hand, Sala *et al.* (1990) found the frequency of salt- and drought-tolerant tomato mutants similar between the chemical mutant combination EMS/MNU and somaclonal variation. Upelniek *et al.* (1995) found a higher frequency (2.07% cf. 0.69%) of mutations affecting wheat grain gliadins from nitrosoethylurea than from cell culture.

Introgressing Alien Genes

'In particular, the phenomenon may be employed to enhance the exchange required in sexual hybrids for the introgression of desirable alien genes into a crop species' (Larkin and Scowcroft, 1981). It is because of our conviction that recombination and rearrangement are at the heart of much somaclonal variation, that our own work shifted to exploiting it in the introduction of alien genes. If cell culture induces non-homologous recombination within a genome, then in the presence of alien chromosomes, it might also enhance alien gene introgression. The alien chromosomes or chromatin might be introduced by wide sexual crossing or by protoplast fusion.

One of the distinguishing features of this approach is that the researcher sets up a genotype (alien chromosome addition line) to target a specific and predesigned improvement by rearrangement. The post-culture screening of regenerant progeny is also specifically designed to identify or select the desired variant.

Barley Yellow Dwarf Virus (BYDV)

BYDV is a serious viral disease of small grain cereals worldwide. Its importance to wheat has often been underestimated due to the subtlety of its primary symptoms, and the fact that it renders wheat susceptible to a number of secondary root and foliar diseases. No adequate resistance has been identified in wheat germplasm itself. We were able to find a number of sources of resistance in related perennial grasses, notably *Thinopyrum intermedium*, commonly called intermediate wheatgrass (Brettel *et al.*, 1988). This species can be crossed to

wheat, but its chromosomes do not recombine meiotically with the wheat genomes. By intercrossing the two species, a line was produced carrying all the wheat chromosomes and only one alien chromosome – the one carrying the virus resistance gene. This was placed into cell culture. After plant regeneration and analysis over a number of generations, eight independent families out of 1200 have been shown to carry the resistance on recombinant wheat/*Thinopyrum* chromosomes (Banks *et al.*, 1995).

Molecular probes which allow recognition of *T. intermedium* chromatin in wheat background, suggest that the translocations analysed to date are still associated with a block of alien chromatin, and can be followed through a breeding programme using restriction fragment length polymorphisms (RFLP) or randomly amplified polymorphic DNA (RAPD). Nevertheless, the segment of alien chromosome carrying the resistance gene is smaller than the arm of the chromosome from which they derive, at least in some of the recombinants (Banks *et al.*, 1995; Hohmann *et al.*, 1996). After backcrossing to recurrent wheat cultivars, it was shown that there was no apparent yield or quality loss associated with the recombinants, and therefore no impediment to using this resistance for new varieties of wheat.

Thus, we have exploited the somatic chromosomal recombination occurring in culture to achieve the transfer of a useful disease resistance gene from an alien chromosome to a recipient crop chromosome. Without this step of culture-enhanced recombination, the resistance does not recombine meiotically with wheat. As a consequence, the BYDV resistance can now be deployed for wheat improvement and new wheat cultivars are being developed.

Asymmetric Lucerne Somatic Hybrids

Surprisingly, few authors have noted the potential synergy between somatic hybridization and somaclonal variation. Protoplast fusion brings together chromosomes of disparate species, albeit for a brief time, in a cell culture environment. Prior irradiation of one parent (the donor) may bias the chromosome loss to the other parent (recipient), but it may be the cell culture environment which enhances the desired prospect of introgressing genes from the donor into the recipient chromosomes. We are currently attempting to exploit this using lucerne (*Medicago sativa*) as the recipient and *Lotus pedunculatus* as the donor. The characteristics of interest in *Lotus* are foliar condensed tannin (for bloat-safety in grazing ruminants) and acid soil tolerance. A collection of over 4000 asymmetric hybrid plants have been produced following the technical approach of our pilot study (Li *et al.*, 1993). These plants have a general morphology like the lucerne parent, though many plants have some degree of morphological variation. RAPD analysis of a sample of plants demonstrated that most have some *Lotus* DNA present (Stoutjesdijk, Larkin and Sale, unpublished and 1995). Although, the screening for tannins and for aluminium tolerance is continuing, some positives have already been identified. The crucial aspect of this work will be the stability of the desired trait. If the governing genes are still on a *Lotus* chromosome, they might be

expected to be unstable. However, if the genes are now on lucerne chromosomes as a result of culture-induced somatic recombination, then stable genotypes should be recoverable. Other examples of this approach include somatic hybrid derived plants of *Brassica* (Liu *et al.*, 1995) and potato (Xu and Pehu, 1993).

Conclusion

Chemical- and radiation-induced mutations as a means to plant improvement were controversial for at least four decades. As we saw earlier, it is now possible to catalogue many hundreds of mutant-derived cultivars that have stood the scrutiny of merit testing and registration in many countries. It remains to be seen, whether the much more recent approach of somaclonal variation will be able also, with time, to catalogue its contribution in similar terms.

We have been able to describe a number of somaclonal variants at the chromosomal and molecular level. However, we are only speculating as to the chain of causal events which led to these genetic changes. This book will give further examples of molecular characterization and speculation regarding causes. It remains to be clarified, how this phenomenon fits into the bigger biological picture of the plant genome and its extraordinary plasticity. This book is a compendium of much of the current understandings and examples which should serve as a basis for students wishing to explore the broader biological picture.

Mutations induced by chemicals, radiation or culture have perhaps receded from the centre stage of plant improvement because of the advent of genetic engineering. It is already possible to isolate a gene, modify it specifically *in vitro*, and return it to the original plant species to achieve a desired mutation. A good example is the research under way to improve barley's utility for beer brewing by increasing the heat stability of β-glucanase (McElroy and Jacobsen, 1995). The protein structural requirements for heat stability have been defined (Fincher, 1994), the native barley gene has been cloned, and the necessary codon changes have been performed by site-directed mutagenesis. These genes are now being transformed into barley. This more heat-stable glucanase is expected to survive the kilning and mashing, and is anticipated to reduce the viscosity of the wort and reduce the glucan hazes in the beer by degrading the glucan polymers. Such experiments are the ultimate in directed mutagenesis. A number of plant resistance genes have recently been cloned. The study of these genes is expanding our understanding of the genetic basis of host plant resistance to diseases and pests. We might anticipate experiments in the next decade in which resistance genes are manipulated *in vitro* to alter their specificity and effectiveness.

However, the current technical difficulties and regulatory constraints have delayed major contributions from genetic engineering for plant breeding. While the power of molecular biology will certainly and eventually impact mightily on crop improvement, it would seem mutagenesis will continue to pay its way in this endeavour upon which so much of our future welfare depends.

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2. Somaclonal Variation: Mechanism and Applications in Crop Improvement

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Introduction

Somaclonal variation refers to the variation arising in cell cultures, regenerated plants and their progenies, and this general term was given by Larkin and Scowcroft (1981). However, other types of variation arise by specific culture of cells or tissues, which include culture of: protoplasts (protoclonal); anthers and microspores (gametoclonal); callus (calliclonal); apical meristem (mericlonal); leaf, stem, root or other somatic tissues (somaclonal). There are different approaches to create somaclonal variation, which include: (1) growth of callus or cell suspension cultures for several cycles; (2) regeneration of a large number of plants from such long-term cultures; (3) screening for desirable traits in the regenerated plants and their progenies, e.g. *in-vitro* selection to select agronomically desirable somaclones for tolerance to various biotic and abiotic stresses using toxic levels of pathotoxins, herbicides, salts, etc.; (4) testing of selected variants in subsequent generations for desired traits; and (5) multiplication of stable variants to develop new breeding lines.

Somaclonal variation has been reported in a large number of plant species; potato, sugarcane, tobacco, tomato, wheat, rice, brassica and others for various agronomic traits such as disease resistance, plant height, tiller number, maturity and for various physiological and biochemical traits (Table 1). Several useful somaclonal variants have been obtained and some of them have been released as cultivars (Table 2). Somaclonal variation thus appears to be an important source of genetic variability. Tissue culture-derived plants show variation which is akin to mutations in tissues and cultured cells. Larkin and Scowcroft (1981) first reviewed the occurrence of somaclonal variation in plant species. Since then several reviews have been published (Semal, 1986; Morrison and Evans, 1987; Karp, 1995; Evans, 1989; Brar and Khush, 1994; Duncan, 1997; Jain *et al.* in this volume). It has been debated whether somaclonal variation is the result of pre-existing genetic differences in somatic cells or is induced by specific components of the medium. Several factors such as genetic background, explant source, medium composition, and age of culture affect somaclonal variation. The exact mechanism of somaclonal variation is not yet understood. The probable causes include changes in karyotype (chromosome number, structure), cryptic changes associated with chromosome rearrangements, somatic gene arrangements, somatic crossing over, sister chromated exchange, DNA amplification and deletion, transposable elements and DNA methylation. Epigenetic changes also occur

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frequently. It is possible that different processes cause such variation in different species or one or several factors operate simultaneously during *in-vitro* culture, resulting in somaclonal variation. Moreover, somaclonal variation can be characterized based on morphological, biochemical (isozymes) and DNA markers such as Randomly Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLPs) and others (Jain, 1997a; Jain *et al.*, 1997b).

The heritable nature of somaclonal variation has been well documented in several crop plants (Table 1). Variation could also arise due to epigenetic changes commonly observed in tissue cultures, but is non-heritable. Since the genetic variability for agronomic traits is either limited or lacking in certain plant breeding programmes, understanding the mechanism of somaclonal variation is highly desirable. However, somaclonal variation is undesirable in plant improvement or multiplication programmes, which require uniformity or 'true-to-type' plants, especially in micropropagation and transgenic plants. Thus, future research should focus on understanding the process of somaclonal variation so that it could be made more precise and directed, thus enabling better control over the extent and type of somaclonal variation. This will further enhance the efficiency of various tissue culture programmes aimed at creating new genetic variability for agronomic traits, as well as for the multiplication of uniform and 'true-to-type' elite germplasm devoid of somaclonal variation.

Factors Influencing Somaclonal Variation

Several factors affect the type and frequency of somaclonal variation, including explant source, genotype, culture medium, and age of the culture.

Explant Source

In-vitro growth may occur from meristem cultures, which may form callus or direct shoot formation. Callus is further differentiated into organized structures by somatic embryogenesis or organogenesis. Departure from organized growth is a key element in somaclonal variation. Generally, the longer the duration of callus/cell suspension in culture phase, the greater the chances of generating somaclonal variation. In several cases, embryogenic cell lines are highly stable and do not exhibit somaclonal variation (Isabel *et al.*, 1993). Jain *et al.* (1995) emphasized that plants regenerated via somatic embryogenesis produce true-to-type progeny and minimize variation. Somaclonal variation can also occur in embryogenic cultures, if they are kept for a long time in cultures, depending upon the plant species.

Genotype

Numerous reports indicate that somaclonal variation is genotype dependent. Differences in the frequency of somaclonal variation have been shown among species or among genotypes within a species. Zehr *et al.* (1987) showed variation in the frequency of segregating qualitative mutations among seven genotypes.

Table 1. Some examples of the heritable somaclonal variation in selected crops

Crop	Trait(s)	Inheritance*	Reference
Tobacco	Resistance to toxin methionine sulfoximine	S	Carlson, 1973
	Resistance to *Pseudomonas syringae*	S	Thanutong *et al.*, 1983
	Leaf colour	S	Dulieu and Barbier, 1982
	Leaf spots	S	Evans *et al.*, 1984
	Tolerance to aluminium	S	Conner and Meredith, 1985
	Resistance to herbicides chlorsulfuron and sulfometuron methyl	S	Chaleff and Ray, 1984
Maize	Resistance to *Helminthosporium maydis* race T	Maternally inherited	Gengenbach *et al.*, 1977
Tomato	Resistance to *Fusarium oxysporum*	S	Evans *et al.*, 1984 Shahin and Spivey, 1987
	Fruit colour	S	Evans and Sharp, 1983
	Resistance to tobacco mosaic virus	S	Cassells *et al.*, 1986
Wheat	Reduced wax, awning, glume colour, gliadin pattern	S	Larkin *et al.*, 1984
	Adh isozyme	S	Brettell *et al.*, 1986
	Resistance to *Helminthosporium sativum*	S	Chawla and Wenzel, 1987
Rice	Resistance to *Xanthomonas oryzae*	S	Ling *et al.*, 1985
Brassica	Resistance to *Phoma lingam*	S	Sacristan,1982
	Seed colour	S	George and Rao, 1983
Alfalfa	Resistance to *Fusarium oxysporum*	S	Hartman *et al.*, 1984
Sugarcane	Resistance to Fiji disease	VP	Krishnamurthi and Tlaskal, 1974
	Resistance to *Helminthosporium sacchari*	VP	Heinz *et al.*, 1977; Larkin and Scowcroft, 1983
Potato	Resistance to *Altenaria solani*	VP	Matern *et al.*, 1978
	Resistance to *Phytophthora infestans*	VP	Shepard *et al.*, 1980
	Mitochondrial DNA restriction pattern	Maternally inherited	Kemble and Shepard, 1984
	Ribosomal DNA copy number	VP	Landsmann and Uhrig, 1985

*S = Sexual transmission; VP = transmission through vegetative propagation.

Usually, it is difficult to separate genotypic effect from differences in tissue culture response, e.g. medium, explant, cultural conditions and their interactions. However, genotype can influence somaclonal variation irrespective of regeneration mode (Bebeli *et al.*, 1988). Somaclonal variation resulting from changes in

chromosome number and rearrangements is easier to recover in regenerants of polyploids than diploids and haploids, since polyploids can tolerate more gross genomic alterations as compared with diploids and haploids. On the other hand, gene mutations are better expressed in haploids and diploids, but show better survival in polyploids, particularly when such changes are deleterious. In any case, some genomes are more stable irrespective of ploidy level. Gonzales *et al.* (1996) suggested that in barley the source of explant, the type of callus (morphogenic vs non-morphogenic) and duration of culture was more important than the genotype in determining the chromosome stability of the cultures.

The late-replicating nature of heterochromatin can result in enhanced chromosome breakage during *in-vitro* culture (Lee and Phillips, 1988). However, Bebeli *et al.* (1993a,b) did not find this relationship. They compared two triticale lines with and without heterochromatin on the long arm of chromosome 7R and the short arm of chromosome 6R. Significant variation was found in R2 but it was most frequent in lines lacking heterochromatin. Different amplification sites may be involved in different genotypes (Reed and Wernsman, 1989).

Genotypes carrying transposable elements are more unstable in culture than those without transposons (Peschke and Phillips, 1991) but not all changes in such lines are due to the movement of transposons (William *et al.*, 1991).

Culture Medium

Somaclonal variation is influenced by growth regulators in the culture medium. It is possible that growth regulators act as mutagens. Auxin 2,4-D (2,4-dichlorophenoxyacetic acid) has been shown to increase the frequency of mutations in colour of stamen hair from pink to blue in *Tradescantia*, and to increase the frequency of sister chromatid exchanges in root-tip cells of *Allium* (Dolezel *et al.*, 1987). Most of the evidence indicates that growth regulators influence somaclonal variation during the culture phase through their effect on cell division, degree of disorganized growth and selective proliferation of specific cell types (Gould, 1984). Even in the absence of the callus phase, deformed shoot primordia have been observed in vegetatively propagated *Kalanchoe*. Similarly, inflorescence variation in micropropagated oil palm could be due to the excessive use of cytokinins.

Age of Culture

Duration of callus culture has a marked effect on the frequency of somaclonal variation. The reduction or even total loss of regeneration ability is a general phenomenon observed in many long-term callus or cell culture lines. Nehra *et al.* (1990) showed that, after 24 weeks, strawberry callus derived from *in-vitro* cultured leaves completely lost its regeneration capacity. Deverno (1995) reported that the frequency of somaclonal variation increased with the duration of *in-vitro* culture, either as callus or cell suspension. Muller *et al.* (1990) found that the level of DNA polymorphism increased with length of time in culture, although chloroplast genome is generally considered to be more highly conserved and stable than nuclear and mitochondrial genomes. However, prolonged culture re-

sulted in the deletion of parts of the chloroplast genome of rice (Kawata *et al.*, 1995). These deletions were associated with changes in plastid morphology. Skirvin and Janick (1976) detected variation in calli clones of *Pelargonium*. Both explant source and age of callus affected the extent of somaclonal variation. McCoy *et al.* (1982) reported a high frequency of cytogenetic abnormalities in cell cultures of oats, such as heteromorphic bivalents, ring chromosomes, tripolar divisions and lagging chromosomes. The frequency of plants showing these variations increased with duration of *in-vitro* culture. The frequency increased from 11% to 50% in the Tippicanoe cultivar as the culture phase extended from 4 to 20 months. Available evidence supports the age effect on the increased frequency of mutation, which is primarily due to sequential accumulation of mutations over time rather than an increased mutation rate in old cultures.

Mechanism of Somaclonal Variation

The mechanism of somaclonal variation is poorly understood. Some of the possible mechanisms leading to somaclonal variation include: (1) karyotypic changes, (2) point mutations, (3) somatic crossing over and sister chromatid exchange, (4) somatic gene rearrangement, (5) DNA amplification, (6) transposable elements, (7) DNA methylation, (8) changes in organelle DNA, and (9) epigenetic variation.

Karyotypic Changes

Cultured cells and regenerated somaclones exhibit changes in karyotype involving both chromosome number and structure (D'Amato, 1985; Lee and Phillips, 1988; Fourre *et al.*, 1997). Numerous examples are available on the occurrence of ploidy changes and chromosome rearrangement such as translocations, inversions, deletions and duplications during tissue culture (Lee and Phillips, 1988). Cryptic chromosome rearrangements could also be a major mechanism to generate somaclonal variation. Small chromosome deletions, inversions and reciprocal and non-reciprocal rearrangements also occur frequently in tissue culture. Such changes in chromosome structure could affect expression and transmission of specific genes by deletion of one copy of a gene or by gene conversion during repair processes. In addition, recombination or chromosome breakage can occur in preferential regions or hot spots of specific chromosomes. Lee and Phillips (1988) consider two main reasons for the high frequency of these changes: late replication of heterochromatin, and nucleotide imbalances that are a consequence of composition of the cell culture medium.

Orton (1980a,b) found enhanced multivalent formation among somaclones derived from the sterile F_1 hybrid, *Hordeum vulgare* × *H. jubatum*. The original hybrid showed no synapsis between the chromosomes of two genomes. In some haploid somaclones, bands between genomes of two species were detected, indicating that some chromosome exchange had occurred, prior to chromosome elimination.

Ahloowalia (1983) reported reciprocal translocations, deletions, inversions, chromosome fragmentation and polyploidy in somaclones of triploid ryegrass. Chromosome rearrangements can affect the gene in which the chromosomal breaks occur; neighbouring genes, particularly those for which transcription may be coordinately regulated, will also be affected. If reunion or transposition to a different site occurs then distant gene functions may also be altered, due to position effect. Cryptic changes not only result in the loss of genes and their functions but also affect the expression of genes which have hitherto been silent. For example, a rearrangement may delete or otherwise switch off a dominant allele, allowing the recessive allele to affect the phenotype.

Point Mutations

This type of variation has been the least reported among the somaclones. Evans and Sharp (1983) reported 13 different single gene mutations among 230 regenerated plants in tomato. These involved both recessive and dominant mutations. Similarly, several single gene mutations have been reported in wheat somaclones (Larkin *et al.*, 1984). Heritable variation was demonstrated for several traits under both simple and quantitative genetic control. Chaleff and Mauvais (1984) obtained a chlorsulfuron-resistant tobacco mutant from cell culture. The mutant was due to a resistant acetolactate synthase and possibly due to mutation in the coding region of the corresponding gene. Several mutants resistant to the herbicides chlorsulfuron and sulfometuron methyl isolated from cell cultures of tobacco were analysed. Resistance was inherited as a single dominant or semi-dominant mutation (Chaleff and Ray, 1984). Shahin and Spivey (1987) obtained somaclones in tomato resistant to *Fusarium* wilt derived from protoplast culture. A single dominant gene conferred resistance to wilt. Conner and Meredith (1985) isolated aluminium-tolerant mutant from protoplast-derived *Nicotiana* whose inheritance indicated a single dominant gene control.

Somatic Crossing Over and Sister Chromatid Exchange

Somatic crossing over was first characterized in *Drosophila* but has also been demonstrated in tobacco, tomato, *Antirrhinum majus, Tradescantia*, soybean and *Gossypium barbadense* (Evans and Paddock, 1976). Environmental factors and certain agents are known to increase the frequency. Some of the somaclonal variation may be explained in terms of the tissue culture environment enhancing the frequency of somatic crossing over.

Homozygous recessive mutants have been recovered in primary regenerants of various crop plants. Non-segregating variants arising directly in the primary regenerants have been reported in tomato, rice, wheat, maize and rapeseed (Evans and Sharp, 1983; Larkin *et al.*, 1984; Kaeppler and Phillips, 1993a,b). Somatic crossing over has been proposed as a possible mechanism for such variants. Tissue culture may enhance the frequency of somatic crossing over. Asymmetric and other exchanges between non-homologous chromosomes could also generate

somaclonal variants. Asymmetric sister chromatid exchange could lead to deletions and duplications which, in somatic cells, would segregate in the subsequent mitotic divisions. In past, breeders have only had access to variation that is normally transmitted through meiosis; the recovery of mitotic crossovers may constitute a unique source of new genetic variation.

Somatic Gene Rearrangement

Somatic gene rearrangement is known to occur in the mouse immunoglobulin genes (Brack *et al.*, 1978; Molgaard, 1980). During differentiation of mouse embryonic cells to plasma cells, the chromosomal genes undergo extensive rearrangement. Various regions of the embryonic DNA are eliminated during ontogeny and the functional regions are 'translocated' together. Similarly during sea urchin ontogeny, there are extensive DNA sequence translocations (Dickinson and Baker, 1978). It is assumed that the germline cells are unaffected. It would be worthwhile to investigate whether such somatic gene rearrangements occur in higher plants; if so, then the regeneration of plants from somatic cells by tissue culture may allow rearrangements to exist in the new germline and may contribute to the occurrence of somaclonal variation in higher plants.

DNA Amplification

Available evidence indicates that some genes in higher organisms can amplify themselves during differentiation or in response to environmental stresses. Depending on how gene expression is regulated, this could mean that the production of mRNA and protein from that gene is increased. The copy number of particular sequences may vary during and after cell culture. This phenomenon has been well studied in flax grown under different environmental conditions. Reassociation kinetics showed that the large plant form (L) had a class of moderately repeated DNA not present in either the small (S) or normal (P1) forms. S had 70% fewer ribosomal cistrons than L or P1.

Such amplification or deamplification of DNA sequence copies may account at least in part for somaclonal variation. Somaclonal variation can cause genetic changes that range from single base pair change (mutation) to more gross chromosomal changes such as deletions, translocations and number. Deamplification of repetitive sequences has been reported in potato (Landsmann and Uhrig, 1985) and wheat and wild barley (Breiman *et al.*, 1987a,b). Amplification has been detected in somaclones for the genes conferring resistance to herbicides in alfalfa (Donn *et al.*, 1984). The T-DNA in transformed cells has also been observed to amplify after prolonged cell culture (Peerbolte *et al.*, 1987).

Alteration of a single base pair resulting in changes of a single amino acid in the polypeptide sequence has been demonstrated in somaclonal variants (Brettell *et al.*, 1986). A reduction in rDNA spacer sequences was observed in one family of regenerated plants. Landsmann and Uhrig (1985) detected deficiencies in ribosomal RNA genes in tissue culture regenerated plants of potato. Such

deficiencies in rDNA have been observed in flax grown under stress environment (Cullis and Charlton, 1981). It is possible that tissue culture stress may be influencing the genome of regenerating plants in a similar fashion to that in flax plants grown under stress environment. Lapitan *et al.* (1988) observed translocations and deletion at the cytological level and at molecular level, and amplification of a 480-bp sequence on the short arm of telomere of chromosome 7R in triticale somaclone. Such changes in gene copy number can alter the phenotype without altering the gene responsible. Arnholdt-Schmitt (1995) also found differences in the copy number of repetitive DNA fragments in regenerants of carrot.

Transposable Elements

Transposable elements are stretches of DNA which can move from one place to another in the genome. The excision and reinsertion of such elements can directly affect the expression of neighbouring structural genes. Moreover, imprecise excision of transposable elements may generate rearrangements of adjacent chromosomal sequences. Transposable elements are known to cause a variety of changes in gene expression and chromosome structure. McClintock (1950) discovered controlling elements (now described as transposable elements) in the progeny of maize plants that had undergone a cycle of chromosome breakage, joining of broken ends, and rebreakage. McClintock (1984) ascribed the release or activation of transposable elements by chromosome breakage to the 'genomic stress' that a broken chromosome causes within a cell.

Peschke *et al.* (1987) made test crosses of 1200 progeny from 301 tissue culture regenerated plants and identified 10 regenerated plants from two independent embryo lines containing an active *Ac* transposable element. No active *Ac* elements were present in the explant sources. Recovery of transposable element activity in regenerated plants indicates that some of the somaclonal variation may be the result of insertion or excision of transposable elements or both. Similarly, the *Spm* element in maize can be activated by tissue culture. Activation of the *Tnt2* transposable element in tobacco suggests that transposable elements are responsible for part of the somaclonal variation.

Chromosome breakage in tissue culture leading to transposable element activation may be due to late-replicating heterochromatin. Late-replicating hetechromatin has been observed in maize, oats and many other species. Late-replicating chromosome regions have been associated with chromosome breakage in cultures of *Crepis capillaris*. McCoy *et al.* (1982) also observed chromosome breakage in tissue culture regenerants of oats near the centromere, a highly heterochromatic region.

Changes in methylation pattern of inactive transposable elements may also be responsible for their activation in tissue culture. Transpositional events in mtDNA have been important in the spontaneous reversion to fertility of S male sterile cytoplasm in maize (Levings *et al.*, 1980). There is physical evidence for the mobility of certain repetitive DNA in yeast. Such change has been correlated with mutational events at the *his4* locus in yeast (Roeder and Fink, 1980).

Transposable elements thus could play a role in somaclonal variation, particularly in a tissue culture environment which could be more conducive to sequence transposition.

DNA Methylation

Phillips *et al.* (1990) hypothesized that variation in DNA methylation could be a principal factor in tissue culture-induced mutagenesis. Methylation changes could affect variation and that could result in chromatin structure alterations. Such alteration may lead to late replication of heterochromatin and therefore to chromosome breakages, and changes in gene expression. Variation in methylation at specific sites could result in changes in gene expression in either a positive (transposable element activation) or in a negative fashion.

Brown *et al.* (1991) found that DNA methylation and base sequence changes are frequent in maize callus and among regenerated plants. Brown *et al.* (1990) and Muller *et al.* (1990) reported a high frequency of methylation and sequence variation among progeny of regenerated rice plants. Significantly increased levels of DNA polymorphism were observed in regenerants as compared with control plants. Analysis by methylation-sensitive and -insensitive restriction enzymes, however, showed that methylation changes cannot be considered as a major factor in the induction of these changes.

The levels of DNA methylation increased in carrot cultures containing IAA and inositol in the medium (Lo Schiavo *et al.*, 1989). Kaeppler and Phillips (1993a) analysed 21 progenies from tissue culture-derived plants of maize inbred A 188 for DNA methylation changes. A high frequency of DNA methylation variation was detected. Both decrease and increase in methylation were observed. Fifteen per cent of the methylation changes were homozygous in the original regenerated plants. These changes were inherited in the progeny. The results showed that demethylation occurred at a high frequency and could be an important cause of tissue culture-induced variation. In contrast to Brown *et al.* (1990) and Muller *et al.* (1990) methylation variation appeared to be more frequent than changes in base sequences. Smulders *et al.* (1995) used five repetitive probes to analyse DNA methylation in tomato. In leaf DNA, the methylation of cytosines varied widely, from no detectable methylation to complete methylation at nearly all sites screened. Only small differences in methylation were found between callus and leaf DNA.

In mammals, methylation patterns can be erased in the germ line (Holliday, 1990). If this is the case in plants, it may explain how a mutation that consists of change in methylation can be somatically stable but revert to wild type in the progeny of regenerated plants.

Changes in Organelle DNA

Changes in mitochondrial genome occur at high frequency as compared with chloroplast genome. One of the classical examples in which such variation has been reported in tissue culture is in cytoplasmically controlled male sterility

(CMS). In maize, sensitivity to the host-specific fungal toxin of *Helminthosporium maydis* race T, the causal agent of Southern corn leaf blight, is associated with Texas male sterile (CMS-T) cytoplasm. In seed-derived plants, these two characteristics are tightly linked and controlled by mitochondrial DNA. Gengenbach *et al.* (1977), among tissue culture regenerants, recovered toxin-resistant plants with reversion to male fertility. Restriction endonuclease pattern of mtDNA showed significant changes in mtDNA of cell culture-derived plants. One mutation to male fertility and toxin insensitivity was the result of frame shift mutation in mtDNA (Wise *et al.*, 1987). Kemble and Shepard (1984) also detected variation in mtDNA of potato somaclones. Since the number of organelles in a developing shoot apex is much higher than in a mature cell, mutation in organelle DNA can be recovered faster. Chloroplast genomes are generally considered to be more highly conserved and stable than nuclear and mitochondrial genomes. However, prolonged culture resulted in the deletion of parts of the chloroplast genome of rice (Kawata *et al.* 1995). These deletions were associated with changes in plastid morphology.

Epigenetic Variation

In several cases, variation arising through tissue culture may be due to epigenetic variation which is not transmitted to the regenerants and their progenies. Cultured cells when exposed to various stresses may result into transient altered expression or modification of traits. Such changes are temporary and do not manifest in the progenies. Some of the epigenetic changes could be due to DNA amplification, DNA methylation or transposable elements.

Recently, Phillips *et al.* (1994) emphasized that somaclonal variation most likely occurs by a stress–response mechanism. Several unstable or non-transmissible variations have been observed which could be due to transposable elements and/or epigenetic modification. These changes occurring in tissue culture could be due to breakdown of normal control processes. The repeat-induced point mutation (RIP) phenomenon could be operative for the origin of variation in tissue culture. There are several similarities between RIP in *Neurospora* and somaclonal variation in plants. RIP could arise in tissue culture in three ways: (i) duplications could initiate the process, (ii) culture medium components could increase the level of sequence methylation with ensuing changes following a RIP-like process or (iii) the genomic balance which inhibits RIP in normal plants could be disrupted. Future research should focus on understanding the disruption or breakdown of the control process in tissue culture so as to enhance or minimize the extent of somaclonal variation in crop plants.

Applications

Tissue culture techniques are becoming important in plant breeding programmes to enhance the selection efficiency and to widen the gene pool of crops. Some of

these techniques include micropropagation, *in-vitro* selection, embryo rescue, anther culture, somaclonal variation, somatic hybridization and transformation. Of these, somaclonal variation occupies a unique position, because it is both an advantage and disadvantage in the tissue culture system.

Increasing Genetic Variability for Agronomic Traits

An important strategy to use somaclonal variation in crop improvement is to culture highly adapted cultivars but lacking in a few characteristics. Application of somaclonal variation in crop improvement has been discussed in several publications (Evans and Sharp, 1986; Brar and Khush, 1994; Karp, 1995). Several useful somaclonal variants possessing resistance to diseases, insects and tolerance to herbicides, have been isolated (Table 2). Notable examples include resistance to Fiji virus, eye spot, and downy mildew diseases in sugarcane (Heinz *et al.*, 1977), resistance to blight in potato (Shepard *et al.*, 1980), resistance to *Helminthosporium* in maize (Gengenbach *et al.*, 1977; Brettell and Ingram, 1979) and resistance to *Fusarium oxysporum* in celery (Heath-Pagliuso *et al.*, 1989) tolerance to herbicides (Chaleff and Ray, 1984; Grant and McDougall, 1995). Similarly, somaclonal variation has been demonstrated for several other traits.

The potential usefulness of somaclonal variation in crops first became apparent in sugar cane. Variation was observed in morphological traits including changes in cytogenetic and isozyme characteristics. Beginning in 1970, somaclones of a number of clones for their reaction to Fiji disease (a leafhopper-transmitted virus) and downy mildew (*Sclerospora sacchari*) were screened. Several somaclones were identified with increased resistance to both Fiji disease and downy mildew (Heinz *et al.*, 1977). Larkin and Scowcroft (1983) initiated cultures from Q101 sugarcane in an agronomically valuable Australian cultivar whose major defect is high susceptibility to eyespot. After more than 8 months in culture, plants were regenerated. Of the 260 Q101 somaclones assayed for their eyespot toxin sensitivity, a high percentage (8.9%) were highly resistant or nearly immune. The results on somaclonal variation in sugarcane demonstrate that it affects many important characters and holds promise for the improvement of varieties, particularly those with single defects.

Shepard *et al.* (1980) argued that it might be simpler, if possible, to selectively improve a popular variety than to create a new one. Screening over 10000 somaclones (protoclones) of potato cv. 'Russet Burbank' showed significant and stable variation in compactness of growth habit, maturity date, tuber uniformity, tuber skin colour, photoperiod and fruit production. Five of 500 somaclones were more resistant to *Alternaria solani* toxin than the parent and, of these, four showed field resistance to early blight. About 2.5% (20 from 800) somaclones screened were resistant to late blight (*Phytophthora infestans*), some of which were resistant to multiple races of the pathogen. These variant somaclones have retained their phenotype through a number of vegetative generations.

A number of reports demonstrate genetic variation among regenerated plants of seed-propagated crops. Devreux and Laneri (1974) used inbred cultivars of

Table 2. Some examples of somaclonal variants possessing improved traits in crop plants

Crop plant	Improved trait(s)	Reference
Sugarcane	Resistance to eyespot, Fiji disease and downy mildew	Krishnamurthi and Tlaskal, 1974; Heinz *et al.*, 1977
	Resistance to eyespot	Ramos Leal *et al.*, 1996
Potato	Resistance to *Fusarium oxysporum*	Behnke, 1980
	Resistance to *Phytophthora infestans*	Behnke, 1979; Shepard *et al.*, 1980
Sweet potato	Darker and stable skin colour (Scarlet)[b]	Moyer and Collins, 1983
Maize	Resistance to *Helminthosporium maydis*	Gengenbach *et al.*, 1977
Tobacco	Herbicide resistance	Chaleff and Parsons, 1978; Chaleff and Ray, 1984
	Resistance to potato virus Y (NC744)*	Chaplin *et al.*, 1980
	Resistance to blue mould (NC-BMR42, 90)*	Rufty *et al.*, 1996
Wheat	Resistance to *Helminthosporium*	Chawla and Wenzel, 1987
	Tolerance to heat/drought stress (KS89WGRC9)*	Sears *et al.*, 1992
	Resistance to barley yellow dwarf virus (BYDV) from *Thinopyrum* sp. (TC5, TC6, TC9)*	Banks and Larkin, 1995
	Tolerance to frost	Dorffling *et al.*, 1993
	Tolerance to salt	Barakat and Abdel-Latif, 1996
Rice	Lysine content	Sharp and Shaeffer, 1993
	Resistance to blast (DAMA)†	Heszky and Simon-Kiss, 1992
	Dwarf, lodging resistant 10% higher yield than the parent variety (Hatsuyme)†	Ogura and Shimamoto, 1991
	Tolerance to salt	Winicov, 1996
Sorghum	Resistance to Fall army worm (GATCCP 100/101)*	Duncan *et al.*, 1991a
	Tolerance to acid soil (GAC102)* (GC103/104)*	Duncan *et al.*, 1991b; 1992
Tomato	High solid (DNAP9)*	Evans, 1989
	Resistance to race 2 of *Fusarium* (DNAP17)*	Evans, 1989
Celery	Resistance to *Fusarium* wilt (UC-T3)*	Heath-Pagliuso *et al.*, 1989
Brassica	Herbicide resistance	Jain and Newton, 1988
	Tolerance to salt	Kirti *et al.*, 1991
Bell pepper	Fruits with fewer seeds (Bell sweet)*	Evans, 1989
Bermuda grass	Fall army worm resistance (Brazos-R3)*	Croughan *et al.*, 1994
Birds-foot trefoil	Resistance to sulphonyl herbicide (H401-4-4-2)*	Grant and McDougall, 1995
Red clover	Regeneration ability (NEWRC)*	Smith and Quesenberry, 1995
Lathyrus sativus	Low neurotoxin	Yadav and Mehta, 1995

*Improved germplasm developed.

†Cultivar released.

tobacco and showed variation in anther culture-derived somaclones. The isogenic lines produced by anther culture were uniform within themselves but there was extensive variation between these lines (somaclones). Some lines showed dry weight yields up to 10% greater than the original cultivar. Attempts to enhance the level of variation by irradiating (5–2000) the flower buds prior to anther culture, did not increase variation.

Burk and Matzinger (1976) used inbred variety 'Coker 139' of tobacco, which had gone through 15 generations of self-fertilization. Dihaploids were derived by anther culture from this highly inbred line. Five spontaneously doubled haploids were present among 41 colchicine-doubled haploids. Significant variability between the somaclonal lines was observed in yield, grade index, days to flowering, plant height, leaf number, leaf length, leaf width, total alkaloids and reducing sugar content. Spontaneously obtained dihaploid lines showed as much variability as those derived through colchicine treatment. There was no significant variation within lines. It is unlikely that there was any residual heterozygosity in the parental plant.

Oono (1978) used rice seeds from a selfed doubled haploid, and examined about 800 somaclones derived from callus initiated from those seeds. Only 28.1% of the plants were considered normal (parental) in all these characters. There was a wide variation in seed fertility, plant height and heading date. Chlorophyll deficiencies were seen in the second generation of 8.4% of the lines, which is a comparable frequency to that expected from X-ray and gamma-irradiation. Sectorial analysis of plants derived from a single seed callus showed that most of the variation was induced during culture and unlikely to pre-exist amongst the seeds used to initiate the culture. It was estimated that mutations affecting these traits were induced in culture at a rate of 0.03–0.07/cell per division.

Somaclonal variation has been observed to affect the mitochrondial genome. Selection for resistance in cultures of T-cytoplasm maize (sensitive to the Southern corn leaf blight T-toxin of *Helminthosporium maydis* Race T) by recurrent sublethal exposure to T toxin resulted in the recovery of toxin-resistant plants. The same plants were also fertile in contrast to the male sterility of the original parent (Gengenbach *et al.*, 1977). Brettell and Ingram (1979) indicated that the frequency of occurrence of the resistant variants was high (35 out of 60) even when toxin was not added to the cultures prior to regeneration. The restored male fertility and toxin resistance were shown to be cytoplasmically inherited.

In-vitro *selection*

In-vitro selection can be used to select agronomically desirable somaclones, particularly where cellular and whole plant responses are correlated. Such correlations exist only in a few systems; two such examples where *in-vitro* selection has been successfully used are with pathotoxins and herbicides. Selection for increased tolerance to specific diseases has been achieved using known toxins or crude culture filtrates. Heinz *et al.* (1977) selected variants from populations of plants regenerated from tissue culture. These somaclonal variants had enhanced resistance to eyespot, Fiji disease and downy mildew. Gengenbach *et al.* (1977)

selected somaclonal variants from culture of maize T-cytoplasm resistant to toxin produced by *Helminthosporium maydis* race T and the disease it caused, southern corn blight. Thanutong *et al.* (1983) obtained variants in tobacco resistant to two pathogens, *Pseudomonas syringae* and *Alternaria alternata* after selecting protoplast-derived callus on medium containing the pathotoxins. Sacristan (1982) obtained plants resistant to *Phoma lingam* from callus and embryogenic cultures of haploid race which survived exposure to the fungal toxin. Hartman *et al.* (1984) selected lines of alfalfa with increased resistance to both the culture filtrate and the pathogen *Fusarium oxysporum* f.sp. *medicaginis*. Heath-Pagliuso *et al.* (1989) obtained somaclonal variants with increased resistance to *Fusarium oxysporum* in celery. Shahin and Spivey (1987) found somaclones without *in-vitro* selection for resistance to *Fusarium* wilt derived from protoplast culture of tomato. In a number of other cases, somaclonal variants resistant to diseases and insects have been obtained without *in-vitro* selection. The findings support the observations that the tissue culture cycle itself can generate genetic variation and that the frequency of somaclonal variants is more or less similar to those where *in-vitro* selection and mutagenesis of cultured cells were used. It is thus possible to develop useful germplasm through somaclonal variation possessing resistance to diseases and insects even if suitable agents for *in-vitro* selection are lacking. Similar to disease-resistant somaclones, herbicide-resistant variants have been obtained following cell culture; chlorosulfuron resistance (Chaleff and Mauvais, 1984) and picloram resistance in tobacco (Chaleff and Parsons, 1978).

Development of 'Elite' Germplasm and Commercial Cultivars

Somaclonal variation with improved traits has resulted in the development of new useful germplasm, and in a few cases, cultivars have been released (Table 2). Sugarcane cultivar Ono, which is resistant to Fiji disease, was developed from susceptible cultivar Pindar (Krishnamurthi and Tlaskal, 1974). A cultivar of sweet potato Scarlet having yield and disease resistance characteristics similar to those of the parent cultivar but with darker and more stable skin colour, was produced (Moyer and Collins, 1983). In tomato, two promising varieties have been developed from cell culture – DNAP-9 having high solid and DNAP-17 with resistance to *Fusarium* race 2 (Evans, 1989). Some somaclonal variants of anther culture origin have been released as a new cultivar in sweet pepper. Bell sweet, a bell pepper variety, was identified which had few or no seeds when grown in several locations over several generations compared to 330 seeds in the control variety, Yolo Wonder (Evans, 1989). Heszky and Simon-Kiss (1992) produced several somaclones in rice and one of these variants was released as a variety named DAMA. This variety is resistant to blast, caused by the fungal pathogen *Pircularia*, and has good cooking quality. Similarly, Ogura and Shimamoto (1991) identified useful somaclonal variants from protoplast-regenerated progenies of Koshihikari, and a new variety Hatsuyume was released. This variety is late by 1 week, shorter in height, lodging resistant, and has 9–11% higher grain yield than Koshihikari. Some 'elite' germplasms possessing tolerance to herbi-

cides, salts and heat/drought stress have been developed through somaclonal variation (Table 2).

Enhancing Alien Gene Introgression into Cultivated Species

The technique of somaclonal variation appears to be particularly important in enhancing variation in interspecific crosses, particularly where the parental genomes of the two species show little or no homoeology. The phenomenon offers tremendous potential for chromosomal exchanges between species which otherwise lack homoeologous pairing. Under such situations, chromosome breakage and reunion could result in new combinations and in the transfer of alien chromosome segments into the cultivated species. A tissue culture cycle of the hybrid material (F_1, monosomic alien chromosome addition or substitution lines, somatic hybrids) could enhance the frequency of genetic exchange. In hybrids of *Hordeum vulgare* × *H. jubatum*, enhanced variation in isozyme pattern and chromosome pairing was observed in contrast to the original hybrid which was asynaptic (Orton, 1980a,b). Cell culture-induced chromosomal exchanges do not seem to rely on homoeology and occur during mitotic cell cycles in the culture rather than meiotic cell cycle. Hence, non-homoeologous and non-reciprocal interchanges are common in tissue culture systems. Lapitan *et al.* (1984, 1988), following cell culture of wheat-rye hybrids, observed chromosome exchanges between 1R and 4D and 3R and 2B.

Larkin *et al.* (1989) and Banks *et al.* (1995) reviewed the usefulness of cell culture to enhance alien introgression in wide crosses. Tissue culture of wheat–rye monosomic addition lines showed introgression of cereal cyst nematode resistance from rye to wheat. Similarly, tissue culture of monosomic alien addition lines of wheat – *Thinopyrum intermedium* showed introgression of barley yellow dwarf virus (BYDV) resistance into wheat (Banks *et al.*, 1995). Of the 1200 plants regenerated from the cultures of monosomic alien addition lines, 14 families were identified in which BYDV resistance was inherited to the progenies. These examples demonstrate that cell culture-induced chromosomal exchanges can be used to transfer alien genes into crop plants. The technique appears equally promising to obtain chromosomal exchanges and derive progenies with introgression of useful genes from somatic hybrids produced through protoplast fusion among widely divergent species.

Improvement in Ornamental Plants

Tissue cultured ornamental plants often show somaclonal variation which may change plant morphology, leaf morphology, flower colour and shape, and leaf variegation (Jain *et al.*, 1997a,b). Jain (1993a,b) reported a wide range of somaclonal variation for flower size, plant height, plant morphology, and number of flowers per plant in *Begonia* × *elatior* plants regenerated from leaf-disc callus. A similar type of variation was observed in plants regenerated from leaf discs of *Saintpaulia ionantha* L. without callus phase. However, no variation for flower colour was

obtained (Jain, 1993a,b). Flower colour variation has been reported in tissue culture-derived plants of carnation (Silvy and Mitteau, 1986), chrysanthemum (Khalid *et al.*, 1989), and gerbera (Buiatti and Gimelli, 1993). Selected somaclones can be further micropropagated to establish the stability of somaclones in the subsequent generations. Jain (1993a,b) observed that selected somaclones of *Begonia* and *Saintpaulia* did not show any variation in the number of flowers per plant in the subsequent two generations. The findings support the theory that somaclonal variation can be exploited commercially in ornamental plants (Duncan, 1997).

Genetic Fidelity and Somaclonal Variation

Somaclonal variation is undesirable in true-to-type large-scale mass propagation, in clonal propagation of woody and ornamental plants, and transgenic plants. The economic disaster can be enormous as a result of somaclonal variation in forest trees and other woody plants, since they have long life cycle. It is thus important to maintain genetic stability in tissue culture-derived woody plants, particularly for reforestation (Jain, 1997b). Some of the genetic changes are difficult to observe at the morphological or physiological level because of the structural difference in the gene product, and that may not alter its biological activity sufficiently to produce an altered phenotype. 'Silent mutations' at morphological and physiological levels are significant since they allow an estimation of the frequency of genomic change as a result of *in-vitro* culture (Sabir *et al.*, 1992). Isozyme and DNA markers can be conveniently used to detect tissue culture-induced variation. Isozyme markers have proven useful to detect somaclonal variation among regenerants in apple stocks (Martelli *et al.*, 1993).

However, Shenoy and Vasil (1992) analysed regenerants of Napier grass derived through somatic embryogenesis. Isozyme analysis showed no variation among the regenerants. Similarly, somatic seedlings of *Picea abies* (Heinze and Schmidt, 1995), *Picea mariana* (Isabel *et al.*, 1993) and in other woody plants showed no variation at the molecular level with RAPD and RFLP markers. Taylor *et al.* (1995) observed limited RAPD polymorphism in sugarcane plants regenerated from embryogenic cultures, indicating infrequent genetic changes during tissue culture. More recently, the amplified fragment length polymorphism (AFLP) technique has become available, offering great potential to analyse genetic variation. This method generates a large number of markers, and may allow the identification of a relatively low level of somaclonal variation.

Advantages and Limitations of Somaclonal Variation

Somaclonal variation offers several advantages: (1) it is relatively cost-effective when compared with other methods, (2) it requires routine laboratory and field facilities, and hence research can be carried out in any plant breeding programme having tissue culture facility, (3) it constitutes a rapid source of genetic variabil-

ity, particularly in crops having a narrow genetic base and which are difficult to improve through conventional breeding, (4) it is successful in removing one or a few defects in otherwise well-adapted cultivars, (5) it improves various vegetatively and seed propagated species, and (6) it produces novel variants.

Plant breeders always look for useful somaclones of practical importance. Somaclonal variation is a good supplement to conventional crop improvement programmes which aim to overcome specific defects in otherwise well-adapted and high-yielding genotypes. However, in several cases somaclonal variants have not advanced beyond the laboratory or greenhouse phase, possibly because the selected material has limited practical value or the trait obtained was not a novel one. Some of the limitations are: (1) poor plant regeneration from long-term cultures of various cell lines; (2) regeneration being limited to specific genotypes which may not be of much interest to breeders; (3) several somaclones are unstable after selfing or crossing; (4) some somaclones have undesirable features such as aneuploidy, sterility etc.; (5) variation is usually not novel; and (6) it is difficult to predict the nature of somaclonal variation.

Future Outlook on Somaclonal Variation

Somaclonal variation has been demonstrated in a large number of vegetatively and sexually propagated species for several agronomic and biochemical traits, and many of them are inherited in the progenies. Epigenetic variation, which is non-heritable, occurs most frequently. The type and frequency of somaclonal variation are affected by genotype, explant source, medium composition, age of the culture, and *in-vitro* culture conditions. Furthermore, changes in karyotype, cryptic chromosome rearrangements, DNA amplification, point mutations, somatic crossing over, somatic gene rearrangements, transposable elements, DNA methylation, and repeat-induced point mutation phenomenon are responsible for somaclonal variation. Numerous somaclones have been reported in crop plants; however, only a few have been used in crop improvement because of low frequency of useful variation, genetic instability, undesirable traits or lack of novelty. Future research should focus on understanding the mechanism of somaclonal variation, so that desirable somaclones could be produced, and on minimizing somaclonal variation for clonal propagation and transgenic research. More emphasis should be given to isolating large numbers of somaclones with changes in one or two desired characteristics which are otherwise lacking in well-adapted cultivars of major crops. Priority should be given to exploiting somaclonal variation in vegetatively propagated species, especially with a narrow genetic base having complex genetic and breeding systems, and those which are difficult to improve through conventional breeding. Enhancing chromosomal exchanges and alien gene introgression through tissue culture of interspecific hybrids and somatic hybrids holds promise, particularly where homoeologous chromosomes lack pairing, and gene transfer through conventional plant breeding methods is difficult. Availability of efficient plant regeneration systems from cultured somatic cells, pollen and protoplasts,

and characterization of somaclones with molecular techniques have widened the potential use of somaclonal variation in crop improvement.

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3. Clonal Variation in Cereals and Forage Grasses

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Introduction

Clonal changes in tissue culture have proved to be a useful method for creating desirable variants in most cereal crops. Variants include changes in physiological mechanisms, agronomic characteristics, plant type, and resistance to pests and stress conditions.

Clonal variation is derived through tissue culturing plant cells or tissues. Clonal variation from somatic tissue explants has been called somaclonal variation. Anther (pollen) or ovary (egg cell) explants give rise to gametoclonal variation. Plants regenerated from callus from protoplast culture show protoclonal variation.

Morphological variations were observed in 1968 when researchers studying rice anther and somatic culture first regenerated plants from varieties (Niizeki and Oono, 1968; Nishi *et al.*, 1968). Ten years later Oono (1978) reported in detail the variation in progeny of selfed rice plants regenerated from leaf tissue culture. Shepard *et al.* (1980) showed extensive variability among plants regenerated from leaf protoplasts from potato. Larkin and Scowcroft (1981a) suggested that somaclonal variation was a useful source of novel variation for plant improvement. Larkin *et al.*, (1984) demonstrated that somaclonal variation in wheat was heritable and stable.

Sources of Clonal Variation

Clonal variation can result from pre-existing genetic variation among cells in the explant tissues and from variation induced during the culturing process (Evans *et al.*, 1984; Karp and Bright, 1985; Larkin and Scowcroft, 1981a; Scowcroft and Larkin, 1988). All of the causes of clonal variation are not yet understood, but known causes include single base-pair changes, chromosome deletions, chromosome rearrangements (Lee, 1988), altered expression of multigene families, changes in ploidy, changes in sequence copy number, activity of transposable elements, interchanges of chromosome fragments, gene amplification, changes in methylation patterns (Brown, 1989; Brown and Lörz, 1986; Larkin, 1987; Larkin *et al.*, 1989), gene conversion (Xie *et al.*, 1995a), and non-random variation (Xie *et al.*, 1995b).

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Pre-existing variation may occur because explants are not normally single-celled; that is, they are tissues or mixtures of tissues and may contain cells differing in ploidy or with variation due to mutation (Skirvin *et al.*, 1994). As plant regeneration in tissue culture often begins from a single cell, these differences are expressed in the regenerated plants.

In tissue culture-induced variation most of the variation appears to occur in the callus phases of the tissue culturing process (Skirvin and Janick, 1976). Callus initiation may be analogous to wound responses in plants which are known to activate transposable elements and to stimulate the induction of stress-induced enzymes and specific by-products (McClintock, 1984). Somaclonal variation also has been related to growth regulators, cultivar variability, cultivar age in culture, ploidy level, explant source, and other culture conditions (Skirvin *et al.*, 1994). The rate of somaclonal variation is a function of all of these factors and has been shown to vary from 0% to 100%. In rice, certain tissue-culture-stable varieties have shown 0% to 1% variation, whereas more unstable varieties showed 10% to 27% variation (Xie and Rush, unpublished). Variability can be increased in tissue-culture-stable rice varieties by using mutagenic chemicals in the culture medium (Xie, 1990).

Useful Clonal Variation in Cereal Crops

Harms (1992) cited variation for disease resistance in wheat, barley, oat, rice, and maize as well as other crops in a review of engineering genetic resistance into crops. *In-vitro* selection for changes in disease resistance usually relies on subjecting cultured cells to selective agents; usually direct exposure to pathogens, culture filtrates from pathogens, or pathotoxins. Oberthur *et al.*, (1993) derived two independently inherited dominant resistance genes for leaf rust from wheat somaclones. These genes were not present in the explant source variety. Somaclonal variation has been reported for mitochondrial DNA variation in plants regenerated from triticale (Weigel *et al.*, 1995) and wheat (Hartmann *et al.*, 1994). Several research groups have reported changes in wheat gliadin proteins due to somaclonal variation (Cooper *et al.*, 1986; Maddock *et al.*, 1985; Obukhova *et al.*, 1991). Other reports suggested that variation in gliadin proteins may come from sources other than somaclonal variation (Metakovsky *et al.*, 1987; Upelniek *et al.*, 1995).

Stable somaclonal variation has been reported in wheat for agronomic traits including spike length, number of grains per spike, 100-grain weight, spike density and biomass, higher grain protein without yield reduction, awns, height, fertility, tiller number, grain colour, heading date, waxiness, glume colour, and other morphological characters. Mutations were observed from both dominant to recessive and recessive to dominant (Carver and Johnson, 1989; Larkin *et al.*, 1984; Maddock and Semple, 1986; Mohmand and Nabors, 1990). Somaclonal variation for dwarfing in wheat was apparently controlled by a single, partially dominant gene which was complementary with the grass-dwarf gene *D1*. It was considered to be a new allele at the *D2* or *D4* grass-dwarfism locus (Guenzi *et al.*, 1992).

In-vitro selection for abscisic acid insensitivity in wheat led to the development of two abscisic acid-insensitive somaclones, KTC86211 and KTC86424, that had significantly delayed senescence and greater kernel weight and grain yield per plant than the parent cultivar. It appeared that abscisic acid-insensitive variants had delayed senescence, higher growth rates, and may have had improved heat and drought resistance (Lu *et al.*, 1989). Other investigations on somaclonal variation in wheat suggested that selection could be successfully made for resistance to the Russian wheat aphid and for freezing tolerance (Lazar *et al.*, 1988; Zemetra *et al.*, 1993).

Culturability and the amount of clonal variability in wheat and other cereal crops has been demonstrated many times to be dependent on the germplasm cultured. In barley, several studies indicated that little variation was observed among somaclones from the varieties used as parents (Baillie *et al.*, 1992; Gozukirmizi *et al.*, 1990; Karp *et al.*, 1987; Luckett *et al.*, 1989). Other investigators have found significant and stable variation in barley (Bregitzer and Poulson, 1995; Ullrich *et al.*, 1991). Many of the variant characters were unfavourable for breeding programmes, including changes in the malting quality of barley somaclones (Bregitzer *et al.*, 1995). Recent research showed that glyphosate tolerance could be significantly increased in barley somaclones regenerated on media containing glyphosate (Escorial *et al.*, 1996).

Bhaskaran *et al.* (1987) reported stable somaclonal variation for several traits in sorghum including reduced height, increased tiller number, increased grain yield, and increased seed number. Seed size was reduced in all of the somaclones. Smith and Bhaskaran (1988) showed that the increased tiller number and reduced height characteristics were still expressed after three generations of selfing. Isenhour *et al.* (1991) showed an increase in resistance to leaf-feeding by the fall armyworm in sorghum somaclones. The resistance in these lines was stable and was expressed when larvae were fed diets containing dried foliage from the resistant somaclones. Waskom *et al.*, (1990) and Miller *et al.*, (1992) produced somaclonal lines from sorghum with significantly improved tolerance to acid soils and drought stress. Variation in seedling survival, panicle size, panicle shape, plant height, seed colour, and plant vigour also were observed. Cai *et al.*, (1990) screened 6000 somaclones from high-tannin sorghums for variation. They observed 43 variant phenotypes that included chlorophyll deficiencies, dwarfing, height reduction, narrow leaves, ragged leaves, multi-branched heads, and *Hydra*, a developmental variant that produces a large number of panicles. Cai *et al.*, (1995) also reported significant, stable increases in polyphenol levels in somaclones from high-tannin sorghum lines.

Dahleen *et al.*, (1991b) reported significant, stable changes in height, heading date, seed protein, flag leaf area, seed weight, seed number, and grain yield in somaclones from oats and their parent varieties. They also reported variation in avenin patterns (Dahleen *et al.*, 1991a).

Variation from rice somaculture has been documented by many researchers (Cao *et al.*, 1991; Oono, 1983; Xie, 1990; Xie *et al.*, 1987, 1990b; Zheng *et al.*, 1989). Protoclonal variation also was reported for rice (Abdullah *et al.*, 1989;

Ogura *et al.*, 1987) and several other crops (Engler and Grogan, 1984; Lörz and Scowcroft, 1983; Shahin and Spivey, 1986; Shepard *et al.*, 1980). The field performance of 125 R_1 plants regenerated from protoplasts of four *japonica* cultivars was reported by Ogura *et al.*, (1987). Chromosomal variants and low seed fertility variants were observed in their experiments. Comparisons of 40 R_2 lines regenerated from Taipei 309 protoplasts with the parent cultivar for 13 quantitative characteristics were reported by Abdullah *et al.*, (1989).

Muller *et al.* (1990) demonstrated that significant DNA alterations occurred among rice somaclones from the same parent. They found a direct correlation between plants with methylation changes and plants with DNA alterations. Changes increased with increasing time in culture, and appeared to be due to the stresses of tissue culture.

Rice plants regenerated from haploid protoplasts derived from pollen callus cell suspension cultures showed stable variation for several traits including height, heading date, and panicle length. Most of the variation was homozygous and fixed (Mezencer *et al.*, 1995).

Homozygous variation from rice somaculture was studied intensively (Xie *et al.*, 1995a,b, 1996). It was concluded that homozygous variation that was stable after crossing arose from non-random variation. Homozygous variation that disappeared or did not segregate normally was apparently lost through unidirectional meiotic gene conversion. Adkins *et al.*, (1990) found stable, significant improvement for drought tolerance in rice somaclones that was germplasm specific and could be improved by *in-vitro* screening of callus on medium with polyethylene glycol. Bertin *et al.* (1995) developed methods for screening rice germplasm for chilling tolerance. Tolerant somaclones were developed and the chilling tolerance was heritable (Bertin *et al.*, 1996). Ling (1991) reviewed the progress of using clonal variation to develop male sterile mutants in rice. Virtually all of the major male sterile phenomena were found as variants in somaclones. Somaclonally derived sterility was more frequent and diversified than that found through crossing or by artificial induction through mutation. Types of male sterility found in somaclones included pollen-free, nuclear gene controlled, pollen-abortive, cytoplasmic-genetic, and antherless/anther-degeneration male steriles. Sun *et al.*, (1991) reviewed the use of somaclonal variation in rice breeding. They indicated that somaclonal variation can be expressed in calli, regenerated plants, and selfed or crossed sexual generations. Clonal variation apparently arises through several different mechanisms, and variant traits can be extensive. Variations could occur alone or together in a plant.

Comparison of Protoclonal and Somaclonal Variation in Rice

The recent development of plant regeneration systems from rice protoplasts (Abdullah *et al.*, 1986; Kyozuka *et al.*, 1987; Thompson *et al.*, 1986; Xie, 1990; Xie *et al.*, 1990a) has provided scientists with an excellent tool for transformation of rice with foreign DNA and for *in-vitro* screening of mutated cells arising

during culture. Protoplast culture provides a large population of individual cells for *in-vitro* selection that is much larger than the plant population normally screened in any breeding programme. Small calli derived from protoplasts in agarose blocks can be exposed to different selection agents, which is ideal for *in-vitro* screening for resistance to pathogens or stress agents. Transformation and *in-vitro* selection research are affected differently by protoclonal variation. For transformation of rice protoplasts with foreign DNA, a low level of protoclonal variation is preferable. High levels of protoclonal variation cause significant problems for transformation research by producing sterile plants and changing important agronomic characteristics. Conversely, the success of an *in-vitro* screening programme relies heavily on variation induced during culture. Knowledge of the type and extent of variation induced by protoplast culture would be helpful for planning research that incorporates protoclonal variation.

Prior to our research, no comparisons of protoclonal variation with somaclonal variation were made. One of our research objectives was to compare protoclonal variation with somaclonal variation in the same cultivar by evaluating and comparing hundreds of protoclones and cell suspension culture somaclones from the cultivar Taipei 309, a tissue-culture-stable cultivar (Xie *et al.*, 1987).

The procedures for plant regeneration from cell suspension and protoplasts of rice were as developed by Xie (1990). Protoclones were compared in the greenhouse with somaclones for the effect of protoplast procedures on variation. No quantitative characteristics were evaluated in this generation (R_1), because of residual effects of the tissue culture process. Eight hundred and twenty R_1 plants regenerated from protoplasts and 340 R_1 plants regenerated from suspended calli plated on solid media were evaluated in the greenhouse for large seed, awned, and sterile variant types. These kinds of changes often indicate chromosomal variation. One hundred and eleven protoclones had one of these variant characters, a frequency of 13.5% variant plants. Only one somaclone generated from suspended calli had the characteristics typical of chromosomal changes, giving a frequency of 0.3%.

Seeds harvested from each fertile R_1 plant were planted in the field along with the parent cultivar at the Louisiana State University Rice Research Station (LSU-RRS) at Crowley, LA in 1990. Somaclonal variation for two quantitative characteristics, days-to-heading and plant height, were recorded and observed qualitative variants were also recorded. For quantitative characteristics, a line was considered variant when it differed from the control at the 1% probability level or if within-line segregation was observed. Four hundred and eighty-seven R_2 protoclonal lines and 298 R_2 somaclonal lines were evaluated in the field for variation.

The effect of protoplast culture on variation for days-to-heading is shown in Table 1. The mean time to heading of protoclones was delayed 11 days compared to the parent cultivar. The mean heading date of somaclones was delayed 2 days. The frequency of protoclonal variants for days-to-heading was 68.6% and 16.7% for the variant somaclones. The variance among the protoclonal lines, however, was slightly smaller than the variance among the somaclonal lines (Table 1). The large difference in the mean number of days-to-heading between protoclones and

Table 1. Effect of tissue culture method on variation for days-to-heading and plant height in the R_2 generation of Taipei 309 rice

Culture method	Number of rows (lines)	Mean*	Variance among lines	Number of variant lines†	Variation frequency (%)
Days-to heading					
Taipei 309 (control)	158	87.1	2.5	0	0.0
Protoculture	478	97.9	21.3	328	68.6
Somaculture	293	89.9	24.1	49	16.7
Plant height					
Taipei 309 (control)	158	105.8	36.6	0	0.0
Protoculture	487	91.1	106.2	68	14.0
Somaculture	298	100.4	50.5	6	2.0

*Mean expressed in days for days-to-heading or in cm for plant height.

†Lines that were significantly different from the Taipei 309 cultivar control ($p \leq 0.01$).

the parent cultivar, the high frequency of variants, and the relatively small variance among the lines suggested that protoplast culture generally increased days-to-heading. The distributions of the lines for days-to-heading are presented in Figure 1. The modes for Taipei 309 and the somaclonal lines were almost the same. The mode for protoclones was 12 days later than those of the parent rows and somaclones.

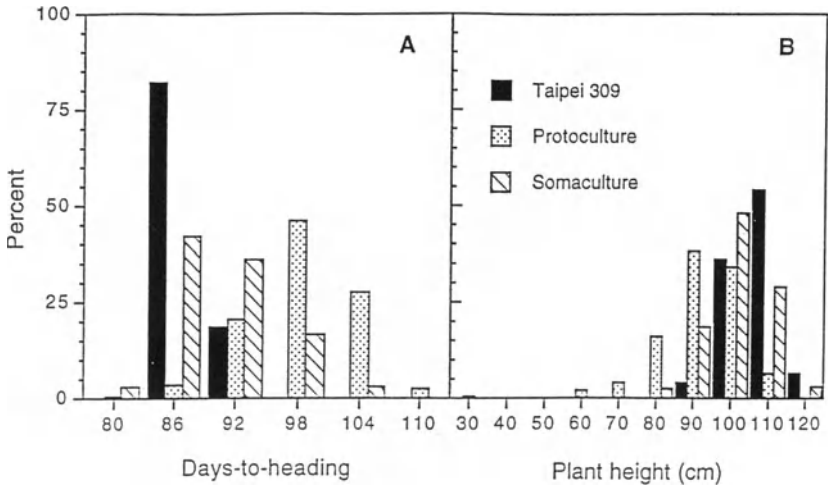

Figure 1. Effect of tissue culture method on percentage distribution of plants from R_2 lines of Taipei 309 rice for days-to-heading **(A)** and plant height **(B)**; the midpoint of each frequency class is shown on the horizontal axis

The effect of protoplast culture on variation for plant height is shown in Table 1. The mean plant height for protoclones and somaclones was reduced by about 15 cm and 5 cm, respectively, when compared with the mean plant height of Taipei 309. The variance was higher for protoclonal lines than for the parent cultivar and somaclonal lines, suggesting that there was more among-line variation caused by protoculture than by somaculture. The frequency of variant protoclones for plant height was 14% and 2% for somaclones (Table 1). The distributions of the R_2 lines for plant height are shown in Figure 1. The distribution of the protoclonal lines was much wider than those of the parent cultivar and the somaclonal lines. The shortest plant among the protoclonal lines was only 30 cm tall, or less than 30% of the mean plant height of Taipei 309. The distributions of the parent lines and the somaclonal lines were similar.

Variation was observed for eight qualitative characteristics among 785 R_2 lines grown in the field (Table 2). Only one kind of variation, albinism, was observed among the 298 somaclonal lines. Variation for seven characteristics was observed among the protoclonal lines, although the variation frequency for each was low.

Our research suggests that protoculture of Taipei 309 had a general effect on plant height and days-to-heading, and may cause unidirectional variation. The same phenomenon was observed by Abdullah *et al.*, (1989). They reported a unidirectional increase in the mean number of days-to-flowering for protoclones. Protoculture appears to have certain general effects on the rice genome. Aside from these general effects, protoculture increased variation frequencies for several qualitative characteristics in this experiment, including glabrousness. The chance of variation from pubescence to glabrousness is slight in rice. Thousands of somaclones from different rice cultivars have been evaluated in our laboratory, and this was the first line showing variation for glabrous leaves from a cultivar with pubescent leaves. This suggests that protoclonal variation has potential for use in mutation breeding, especially when the protoplast regeneration procedure is incorporated into an *in-vitro* screening system. Cell lines resistant to α-picolinic acid

Table 2. Effect of tissue culture method on variation frequency for eight variant characteristics in the R_2 generation of Taipei 309

Variation	Protoculture variation (%)	Somaculture variation (%)
Albinism	0.00	1.34
Yellow leaf	0.83	0.00
Apiculus colour	0.21	0.00
Awn production	0.21	0.00
Wither*	0.42	0.00
Plant type	1.00	0.00
Glabrous	0.21	0.00
Genetic leaf spot	0.63	0.00

*Term used to describe the characteristic where young plants suddenly blight and shrivel.

and phenylacetic acid were generated in our laboratory through *in-vitro* selection of rice protoplast-derived calli (Xie and Rush, 1992), and fertile plants resistant to α-picolinic acid were recently regenerated (Xie and Rush, unpublished). However, the higher frequency of clonal variation caused by protoculture, especially the high frequency of sterile plants in the R_1 generation, made selection of fertile α-picolinic acid plants more difficult. This also has been a problem in transformation of rice protoplasts with foreign DNA. The treatments involved in transformation, such as suspension of protoplasts in PEG solution and screening of protoplasts to antibiotics, made the problem even worse. In some of our transformation experiments, more than 50% of the protoplast-derived R_1 plants were sterile (Xie and Rush, unpublished). Different cultivars react differently to protoculture. Certain cultivars, such as Nipponbare, were more stable in protoculture than Taipei 309 (Xie and Rush, unpublished). Screening and using protoculture-stable cultivars for transformation research could be one solution to this problem (Ogura *et al.*, 1987). A tendency for variation is necessary for *in-vitro* selection, and loss of some regenerants from sterility or other undesirable characteristics must be expected.

Clonal Variation in Rice for Sheath Blight and Blast Resistance

One of the main problems in rice production worldwide is the damage caused by diseases. According to Klaus Lampe, Ex-Director General of the International Rice Research Institute, the value of losses from diseases in 1987 was about 12.5 billion US dollars, not counting the costs of disease control chemicals and their application (Lampe, 1987). Currently, blast, caused by *Pyricularia oryzae*; bacterial leaf blight, caused by *Xanthomonas campestris* pv. *oryzae*; and sheath blight, caused by *Rhizoctonia solani*, are the three most important non-viral rice diseases in the world. In the southern United States, especially in Louisiana, the major diseases of rice are sheath blight and blast (Groth *et al.*, 1991). Development of effective measures to control these diseases is very important to rice farmers.

Historically, the most successful approach for disease control is developing disease-resistant cultivars. Breeding for resistance to rice blast has been carried out since the 1920s (Gangopadhyay and Padmanabhan, 1987). More than 13 major resistance genes have been identified (Ou, 1985), and a large number of resistant cultivars have been released during the past two decades (Gangopadhyay and Padmanabhan, 1987). Most US cultivars also have genes that confer resistance to the major races of *P. oryzae*. However, due to pathogenic variability and instability of the blast fungus (Ou, 1985), the potential of the pathogen to produce new virulent races, or of minor virulent races becoming prevalent, has been a continuing threat to rice production. A continuous supply of new genes for resistance to rice blast has to be made available to the rice breeders.

In recent years, rice sheath blight has become a major problem in most rice-growing regions due to the increased usage of direct seeding, high rates of nitro-

gen fertilizers, and the cultivation of modern semi-dwarf, high-yielding cultivars (Ou, 1985). In the Southern United States, sheath blight has been endemic for many years, and recently has become a serious problem in rice production in Louisiana, Arkansas, and Texas, especially in the US long-grain cultivars (Groth *et al.*, 1991; Lee and Rush, 1983; Marchetti, 1983). No commercial rice cultivar in the world is completely resistant to this disease. All of the US long-grain cultivars except Katy, a moderately susceptible cultivar released in 1989, are highly susceptible to sheath blight. The problem in breeding rice cultivars for resistance to sheath blight is the difficulty in finding suitable sources of resistance. Only a few cultivars are highly resistant and none is completely resistant. The inheritance of this partial resistance is complicated (Sha, 1987). In addition, the agronomic characteristics of these resistant cultivars, for example Tetep and Taducan, are extremely poor. It is difficult to use these sources of resistance in a breeding programme and expect to quickly develop resistant cultivars with good agronomic characteristics. However, there is an urgent need for new long-grain rice cultivars with resistance to sheath blight. A new source of major gene-controlled resistance with acceptable agronomic characteristics would be very valuable in breeding new cultivars with sheath blight resistance. The long-term objectives of our research programme are to generate resistance to blast and sheath blight through clonal variation and to incorporate the resistance into US long-grain cultivars.

Since Carlson (1973) first demonstrated that disease resistance could be generated by tissue culture and selected by screening *in vitro*, plants resistant to several pathogens have been regenerated and selected in 19 different crop species by *in-vitro* screening or directly screening the somaclones (van den Bulk, 1991). Examples are maize plants with resistance to T-toxin (Brettell and Thomas, 1980), sugarcane plants with resistance to eyespot disease caused by *Helminthosporium sacchari* (Larkin and Scowcroft, 1981b), rice plants with resistance to brown spot disease caused by *Helminthosporium oryzae* (Ling *et al.*, 1985), wheat plants with resistance to *Fusarium* (Ahmed *et al.*, 1991), pearl millet plants with resistance to downy mildew caused by *Sclerospora graminicola* (Nagarathna *et al.*, 1993), and tomato plants with resistance to Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici*, race 2 (Shahin and Spivey, 1986). The results indicate that somaclonal variation has the potential to create novel disease resistance. This kind of resistance would be extremely valuable where sources of resistance are not readily available, such as in the case of rice sheath blight, or where a continuous supply of new resistant material is needed for effective disease control, such as with rice blast.

Efficient plant regeneration systems from either rice immature panicle-derived calli or protoplasts have been developed in our laboratory (Cao *et al.*, 1991; Xie *et al.*, 1990b). In the past 10 years, more than 20 000 somaclones and protoclones have been regenerated, and many genetic variations have been observed (Cao *et al.*, 1991; Xie *et al.*, 1987, 1990b, 1995a; Xie and Rush, 1992). Somaclonal variation included differences in morphological characteristics such as colour of apiculus, hull, leaf or sheath; awn production, and changes in leaf pubescence. Variation also has been observed in physiological characteristics such as tolerance

to the fungus toxin, and picolinic acid. Differences were also observed in important agronomic characteristics such as plant height, plant type, tillering ability, panicle shape, grain type and quality, grain weight, kernel numbers, sterility, days-to-heading, and yielding ability; and in resistance to major rice diseases such as sheath blight and blast. Not only has rice somaclonal variation had a wide spectrum, but also a very high variation frequency. A 27% variation frequency was observed in somaclones (Xie *et al.*, 1987) and about 80% variation frequency was observed in protoclones (Xie and Rush, 1994). The ability to induce wide-spectrum variation at high frequency coupled with an efficient plant regeneration system are pre-requisites for a genuine effort to develop new sources of disease resistance through somaclonal variation.

Regeneration, Screening, and Field Performance of Sheath Blight-Resistant Somaclones and their Progeny

The somacultural procedures used were modified from Cao *et al.* (1991). Immature panicle explants were plated onto modified MS callus induction medium (Murashige and Skoog, 1962) with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose and solidified with 8 g/L agar. The panicle pieces were incubated at 28°C in the dark. Calli 2–4 weeks old were transferred to modified MS plant regeneration medium supplemented with 0.5 mg/L indoleacetic acid (IAA) and 0.8/L 6-benzylaminopurine (6-BA) without 2,4-D for plant regeneration. Plants (R_1) were grown to maturity in the greenhouse. Emerging panicles were covered with crossing bags. Seeds of one panicle harvested from each R_1 plant were planted in single rows (R_2) in the field to screen for disease resistance.

In 1986, 1400 somaclonal lines (R_2) regenerated from the sheath blight-susceptible cultivars Labelle and Lemont were planted in the field at the LSU-RRS. Rows were inoculated with *R. solani* isolate LR172 grown on a sterilized, mixture of rice grain and rice hulls (1 : 2 V : V). The somaclone SC 86–20001, regenerated from Labelle, showed a high level of sheath blight resistance along with acceptable agronomic characteristics such as resistance to lodging, glabrous leaves, typical US long-grain quality, good plant type, and excellent ratooning ability. This somaclone was further evaluated for sheath blight resistance in the greenhouse. The results showed that SC 86–20001 had a sheath blight resitance level as high as that of the resistant cultivars Tetep and Taducan (Xie *et al.*, 1990b). In the greenhouse test the somaclone SC 86–20001, Tetep, and Taducan were all rated 3, based on the 0–9 disease rating scale developed by Hoff *et al.*, (1976) with 0 equal to no disease, and 9 equal to plants dead at maturity. The parent cultivar Labelle was rated 9 in the same experiment.

In 1987, 40 panicle rows from SC 86–20001 were tested again in the field along with the parent cultivar Labelle. After inoculation with *R. solani*, the rows were rated at maturity for sheath blight. The average disease rating for SC 86–20001-derived panicle rows was 1.4, and the parent cultivar Labelle was rated 7.5. All 40 panicle rows tested were more resistant than the parent cultivar. However, there were some differences in sheath blight resistance among the SC

86–20001-derived panicle rows with a 0–4 distribution of disease rating[5] among them.

In 1988, two selections from the SC 86–20001-derived panicle lines, SC 86-20001-5 with a 3 disease rating and SC 86–20001–33 with a 0 disease rating in the 1987 field test, along with the parent cultivar Labelle, the susceptible cultivar Lemont, and the resistant cultivar Taducan were evaluated in a replicated field test for sheath blight resistance (Xie *et al.*, 1990b). Each plot was inoculated with *R. solani* and disease ratings were made at maturity. This test further confirmed that the resistance of the somaclonal lines was significantly higher than that of the US long-grain cultivars Labelle and Lemont. The resistance level of SC 86–20001–33, rated 0.8 in this test, was at least as good as that of Taducan, the most resistant cultivar known which rated 1.0 in the same test. SC 86–20001–33 had better agronomic characteristics than Taducan and Tetep. The other somaclonal line, SC 86–20001–5, was rated at 4.8. Lemont was rated 8.7, and the parent cultivar Labelle was rated 9.0. Our study on the inheritance of resistance from these two lines indicated that a single recessive gene controlled the sheath blight resistance of SC 86–20001–5, and two pairs of independently inherited recessive genes were involved in the control of sheath blight resistance of SC 86–20001–33 (Xie *et al.*, 1990b). The somaclonal lines SC 86–20001–5 and SC 86–20001–33 were registered as elite lines for sheath blight resistance in 1992 (Xie *et al.*, 1992). SC 86–20001–5 was named LSBR-5, and SC 86–20001–33 was named LSBR-33.

In 1990, LSBR-5 and LSBR-33 were included in the preliminary yield test in the breeding programme at the LSU-RRS. In this experiment, the yields of LSBR-5 and LSBR-33 were significantly higher than those of the control cultivar Lemont and the parent cultivar Labelle (Table 3) due to severe sheath blight from naturally occurring inoculum. Severe lodging of Labelle, mainly due to weakened culms, also was caused by sheath blight pressure. However, the milling yields of LSBR-5 and LSBR-33 were lower than those of Lemont and Labelle.

In 1991, LSBR-5 was included in the LSU-RRS breeding programme's advanced yield test. The sheath blight pressure was not very severe in this test, and the main crop yield of LSBR-5 was lower than those of the newly released cultivars Cypress and Lacassine (Table 3). The total yield of LSBR-5, due to a good ratooning ability, was similar to that of Lacassine (Table 3). However, the milling of LSBR-5 was again lower than that of the commercial cultivars. Based on these tests, the main characteristics of LSBR-5 and LSBR-33 as elite US long-grain sheath blight-resistant lines are summarized as follows: (1) good field resistance to sheath blight; (2) acceptable yielding ability; (3) good ratooning ability; and (4) poor milling.

Important characteristics of disease-resistant germplasm include the level of resistance of the germplasm and the combining ability of the resistant germplasm with other cultivars. Combining ability becomes even more important when the resistance is created by somaclonal variation, as not all somaclonal variation is stable when crossed with other cultivars (Oono, 1985; Xie and Rush, 1990; Xie *et al.*, 1995a).

Table 3. Agronomic performance of LSBR-5, LSBR-33 and selected cultivars in a 1990 preliminary yield test and a 1991 advanced yield test, Louisiana State University Rice Research Station, Crowley, LA

Entry	Seedling vigour*	Days to heading	Lodging (%)	Yield (kg/ha)†			Milling (%) (W – T)‡
				Main	Ratoon	Total	
Preliminary yield test – 1990							
LSBR-5	5	84	0	–	–	7322[a]	46–65
LSBR-33	6	83	0	–	–	6352[a]	51–63
Lemont	6	85	0	–	–	5750[b]	50–72
Labelle	5	70	85	–	–	3279[c]	55–70
Advanced yield test – 1991							
Cypress	5	85	0	6991[a]	2555	9546[a]	62–70
Lacassine	5	86	0	6507[a]	1988	8495[b]	56–72
LSBR-5	7	91	0	5382[b]	3271	8653[b]	53–65

*Subjective rating of 1–9, where 1 = excellent, 9 = poor.

†Within tests and columns, means followed by the same letter do not significantly differ (Duncan's MRT, $p \leq 0.05$).

‡W = whole grain, T = total grain.

To study the usefulness of this new sheath blight resistance, LSBR-5 and LSBR-33 were crossed with the cultivars Labelle and Lemont in 1987. Panicle rows of successive generations from these crosses were grown through the late 1980s and early 1990s, and selected for sheath blight resistance, agronomic acceptability, and yield potential. Selected lines were passed on to the LSU-RRS breeding programme for yield testing and further selection.

In 1992, four segregates from crosses between LSBR-5 and Lemont were included in the Cooperative Uniform Regional Rice Nursery (URN), along with the LSBR-5 line, as entries from the LSU-RRS rice breeding programme. These lines were assigned the URN numbers 71, 122, 125, and 149. Data on yield and percentage head rice were extracted from the mean data across the four Southern states of Arkansas, Louisiana, Mississippi, and Texas. The mean yields and head rice yields for the four breeding lines, along with several check cultivars, are listed in Table 4. The URN 71 and 149 lines yielded well, but head rice yields were low. Data indicated that the lines were highly to moderately resistant to sheath blight (Table 4). URN 71 was the least resistant of the four lines, but had sheath blight resistance about equal to the medium-grain cultivar Rico 1, which is considered to be resistant in the field.

An additional nine breeding lines from these crosses, previously grown in yield tests by the LSU-RRS breeding programme, and LSBR-5 were compared with the sheath blight-susceptible cultivars Labelle, Lemont, Lacassine, and Cypress for the effects of sheath blight on yield. The cultivar yields in non-inoculated plots were high, ranging from 8665 to 9559 kg/ha. The same cultivars had yields ranging from 6720 to 8718 kg/ha in inoculated plots. Yield losses to sheath blight ranged from 998 to 2322 kg/ha among the commercial cultivars. Yields in non-

Table 4. Yield performance and sheath blight ratings of LSBR-5-derived breeding lines and selected control cultivars as abstracted from the 1992 Cooperative Uniform Regional Rice Nursery results.

Entry	Pedigree	Mean yield (kg/ha)	Mean head rice (%)	Sheath blight rating*				
				AR	TX	MS	LA	Mean
URN 71	Lemont/LSBR-5	8197	55	4.0	7.0	3.7	5.0	4.9
URN 122	Lemont/LSBR-5	6985	54	2.8	5.0	3.0	4.0	3.7
URN 125	LSBR-5/Lemont	6583	57	3.0	2.0	3.0	2.0	2.5
URN 146	LSBR-5	6739	55	0.8	3.0	2.3	3.0	2.3
URN 149	LSBR-5/Lemont	8302	56	3.8	6.0	3.0	4.0	4.2
URN 55	Lemont	7562	63	8.0	8.0	5.7	6.0	6.9
URN 57	Lacassine	8073	60	7.8	9.0	5.3	7.0	7.3
URN 59	Cypress	7865	63	6.5	8.0	4.7	6.0	6.3
URN 118	Labelle	6612	60	8.5	9.0	4.3	5.0	6.7
URN 79	Rico 1	8416	65	5.0	6.0	4.0	4.0	4.8

*Based on a 0–9 rating where 0 = no disease and 9 = plants killed at maturity; data abstracted from tests in Arkansas (AR), Texas (TX), Mississippi (MS), and Louisiana (LA).

inoculated plots of the breeding lines ranged from 7702 to 9270 kg/ha. Yields in inoculated plots ranged from 7214 to 9063 kg/ha. Yield losses to sheath blight ranged from 94 to 842 kg/ha. LSBR-5 and two other breeding lines had higher yields in the inoculated plots than in the non-inoculated plots (Rush *et al.*, 1992). In this test, sheath blight ratings of the breeding lines ranged from 1.3 to 4.8 on the 0–9 scale. Sheath blight ratings of the commercial cultivars ranged from 7.3 to 9.0 (Rush *et al.*, 1992). Breeding lines were much more resistant to sheath blight than were the cultivars, and five lines had a resistance level similar to that of LSBR-5.

In 1993, the breeding line URN 152, from the cross of Lemont and LSBR-5, was included in the URN in addition to URN 71 and 149. All three lines yielded in general with the commercial cultivar checks and had both sheath blight and blast resistance. The three lines yielded well in tests planted in five parishes in Louisiana by the LSU-RRS breeding programme (Table 5). In general, the lines yielded with Cypress, the best long-grain cultivar available (Table 5). The ratoon potential of these lines also was excellent in these tests (Table 6).

In conclusion, the resistance to sheath blight expressed in LSBR-5, LSBR-33, and the breeding lines derived from crosses between these somaclones and Lemont is of sufficient magnitude to be useful in rice breeding programmes. The resistance is stable and has not decreased since the somaclonal line was first tested for sheath blight resistance in 1986.

Regeneration, Screening, and Field Performance of Blast-resistant Somaclones and their Progeny

Regeneration of somaclones from immature panicles of Newbonnet rice (Narvaez, 1992) followed procedures modified from Cao *et al.*, (1991) as described for

Table 5. Main crop yield of three LSBR-5-derived breeding lines and the cultivar Cypress in five Louisiana parish tests in 1993.

Entry	Yield (kg/ha)					Mean
	Acadia	East Carroll	Morehouse	Rapides	Vermilion	
URN 71[a]	8620	8035	9193	6750	8077	8135
URN 149[a]	8219	8941	9199	7568	8542	8494
URN 152[a]	8434	9959	9857	7170	7937	8671
Cypress	8897	9349	10 189	7209	8322	8793

[a]Lines URN 71 and URN 149 were from the cross LSBR-5/Lemont; line URN 152 was from the cross Lemont/LSBR-5.

Table 6. Yields of main and ratoon crops of breeding lines derived by crossing LSBR-5 and Lemont and the cultivar Cypress in two Lousiana parish tests in 1993.

Entry	Acadia Parish			Vermilion Parish		
	Main (kg/ha)	Ratoon (kg/ha)	Total (kg/ha)	Main (kg/ha)	Ratoon (kg/ha)	Total (kg/ha)
URN 71	8620	1682	10 302	8077	5358	13 435
URN 149	8219	1805	10 021	8542	4904	13 446
URN 152	8434	1608	10 042	7937	4380	12 317
Cypress	8897	1114	10 011	8322	4058	12 380

production of sheath blight-resistant somaclones, except that 4 mg/L of 2,4-D were used. At the four-leaf stage, plants (R_1) from tissue culture were transplanted into pots in the greenhouse, where they were allowed to grow to maturity.

Seeds harvested from each R_1 plant were planted in a panicle row (R_2) in the field at the LSU-RRS, and evaluated for disease resistance (leaf blast, rotten-neck blast, panicle blast) and agronomic traits (days-to-heading, height, leaf area, apiculus color) during 1990. The following year, the R_3 generation of each somaclone was planted in five 2-m rows in a randomized complete block design, and the same parameters were evaluated along with plot yield. During both years of the test, the field trial area was flooded when plants reached the three- to four-leaf stage (Narvaez, 1992). The majority of the 112 somaclones tested were more resistant to leaf blast and rotten-neck blast (Fig. 2) than was Newbonnet in both years of the test. In 1991 almost all of the somaclonal lines had greater resistance to panicle blast than did Newbonnet. The majority of the lines matured earlier and were taller than the parent variety Newbonnet in both the R_2 and R_3 generations. Though the parent apiculus colour was purple, 30–40% of the lines showed somaclonal variation for straw-coloured apiculus. Most R_2 lines had larger leaf area than Newbonnet, though this character was not evaluated in the R_3 generation.

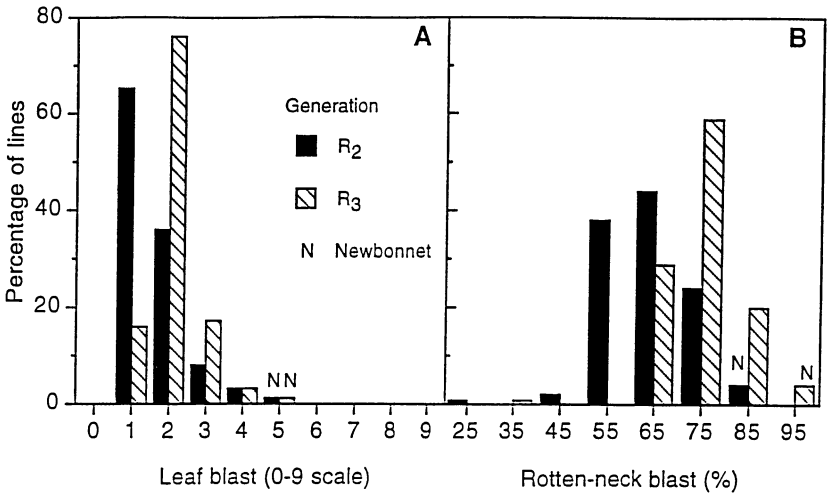

Figure 2. Distribution of mean leaf blast ratings (**A**) and rotten-neck blast ratings (**B**) for Newbonnet R_2 and R_3 somaclonal lines and the parent cultivar evaluated in field trials at the Louisiana State University Rice Research Station, Crowley, LA in 1990 and 1991; the midpoint of each frequency class is shown on the horizontal axis

Newbonnet yields averaged around 108 g per plot in the 1991 field test, while the majority of the somaclones yielded between 140 and 160 g per plot.

Narvaez (1992) also evaluated the same set of Newbonnet somaclones for resistance to leaf blast and rotten-neck blast under non-flooded conditions. These experiments were conducted in 1990 and 1991 in irrigated nursery beds of sandy soil. Leaf blast resistance of 83 R_2 somaclonal lines and 110 R_3 somaclonal lines tested was improved over that of the parental variety for the majority of the lines (Fig. 3). The 83 R_2 lines evaluated in nursery beds had a lower percentage of panicles with rotten-neck blast than did Newbonnet (Fig. 3). The resistance of the somaclonal lines did not appear to be significantly altered by either flooded or non-flooded growing conditions.

Somaclonal Variation in Forage Grasses

Somaclonal variation can be a useful tool for the improvement of forage crops. Screening for traits such as pest resistance can be conducted within large populations of regenerated plants (Croughan, 1989). Many important forage grasses are apomictic, producing progeny that are exact replicas of the female parent. This limits the variability that can be found within natural populations or produced through conventional breeding. Apomixis is a mode of reproduction that can be useful in plant breeding programmes for the development of true-breeding hybrids (Hanna and Bashaw, 1987), but is a barrier to genetic improvement through hybridization. Variability in apomictic species usually is limited to selection of

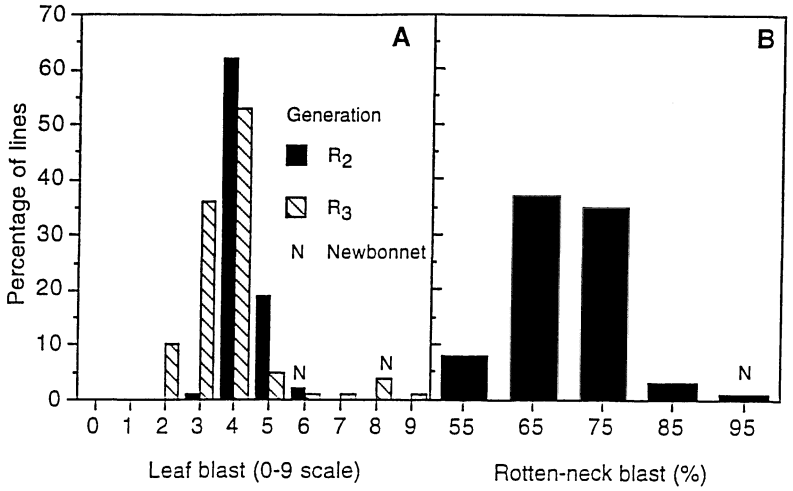

Figure 3. Distribution of mean leaf blast ratings **(A)** for Newbonnet R_2 and R_3 somaclonal lines and the parent cultivar and rotten-neck blast ratings **(B)** for Newbonnet R_3 somaclonal lines and the parent cultivar evaluated in nursery beds at the Louisiana State University Rice Research Station, Crowley, LA in 1990 and 1991; the midpoint of each frequency class is shown on the horizontal axis

ecotypes. Somaclonal variation provides a method to introduce new and possibly unique genetic improvements into apomictic species.

A large number of forage grasses have been regenerated, such as ryegrass (*Lolium* spp.), napier grass (*Pennisetum purpureum*), several *Panicum* spp., bluestem (*Poa pratensis*), orchardgrass (*Dactylis glomerata*), several *Festuca* spp., bahiagrass (*Paspalum notatum*), and bermudagrass (*Cynodon dactylon*). Several reviews in the 1980s discussed the status of forage grass tissue culture (Ahloowalia, 1984; Ozias-Akins and Vasil, 1988; Vasil and Vasil, 1986). Other forage species have been regenerated since, including dallisgrass (*Paspalum dilatatum*). While regeneration systems are well established for many forage species, evaluation for somaclonal variation has been limited.

Variation in traits such as plant morphology, leaf shape and size, spikelet size and shape, floral development, vigour, and survival have been observed in *Lolium* somaclones (Ahloowalia, 1983; Jackson and Dale, 1989). Chromosome alterations, including changes in number, also were observed but not always correlated to phenotypic changes. Bajaj *et al.*, (1981) observed changes in leaf shape in *Panicum* somaclones. Davies and Cohen (1992) evaluated variation in dallisgrass somaclones. Dwarfing, poor vigour, and floral abnormalities were seen in most regenerated plants. Plants tended to have narrow leaves and reduced cold tolerance. A second series of regenerated plants produced after a shorter culture period did not show all the abnormalities observed in the first set of somaclones that were cultured longer. Few of the dallisgrass somaclones expressed improvement in agronomic traits.

Croughan *et al.*, (1995) are evaluating dallisgrass somaclones under field conditions. Approximately 1000 new somaclones are field-tested each year. Traits such

as grazing tolerance, persistence, and ergot infestation are rated. The best 100 somaclones are selected for determination of forage quality, yield, and seed production characteristics. In contrast to the results of Davies and Cohen (1992), we have found improvements in agronomic traits in the somaclones. Seeds of promising lines are being increased for advanced testing.

Davies and Cohen (1992) found alterations in apomictic seed production among dallisgrass somaclones, and progeny derived from some somaclones were not uniform in phenotype. In contrast, Taliaferro *et al.*, (1989) found uniform expression of somaclonal variation in the progeny of somaclones, confirming genetic transmission of somatic variation in floral morphology, plant height, foliage colour, and fertility in apomictic bluestem (*Bothriochloa* sp.) somaclones.

The production of somaclones with specific chromosome changes could be useful for genetic studies. Production of plants with higher ploidy levels can be used in transferring characteristics between species (Park and Walton, 1989). Aneuploids and other genetic abnormalities can be used in genetic mapping studies. For example, variation in isozyme patterns in somaclones can be correlated to chromosome abnormalities (Humphreys and Dalton, 1991). Chromosomal variation in regenerated tall fescue (*Festuca arundinacea*) plants has been extensively studied by a number of researchers (Conger *et al.*, 1980; Dahleen and Eizenga, 1990; Eizenga, 1987, 1989; Reed and Conger, 1985). High rates of chromosomal abnormalities were observed, and the frequency of abnormality increased with time in culture.

Not all studies of forage grass somaclones have detected variation. Several researchers have observed uniformity in regenerated plants. Hanna *et al.*, (1984) regenerated guinea grass (*Panicum maximum*) and selected 20 somaclones to evaluate. These plants had been regenerated after a short time in culture on low levels of auxin. No morphological or cytological differences were observed. Shenoy and Vasil (1992) found uniformity in biochemical and molecular analysis of 63 regenerated plants of napier grass (*Pennisetum purpureum*). Plants also have been regenerated after a short time in culture on relatively low levels of auxins. Valles *et al.*, (1993) regenerated 82 meadow fescue (*Festuca pratensis*) plants from 20-week-old suspension cultures and over 40 plants from protoplast cultures derived from the suspension cultures. Approximately 10% of the regenerated plants were albino or chimaeric (green/albino) and thought to be derived from variant cells in the cultures. The remaining plants appeared phenotypically uniform. Sixteen plants were used for chromosome counts and all found to have $2n = 14$. Molecular analysis (restriction fragment length polymorphism, RFLP, and random amplified polymorphic DNA, RAPD, markers) of the somaclones did not detect any genetic variation among the regenerated plants.

The differences in amount of variability could be related to genotype or species, explant source, or be based on type and length of culture conditions. Croughan and Colyer (1994) found differences in the amount of variation among bermudagrass somaclones tested for leaf spot resistance. Some bermudagrass genotypes produced somaclones with little variation, while others were

significantly different from the parent cultivar. A long stage of callus growth, or a high level of auxin in the callus induction and growth media, also can lead to more variability in regenerated plants. For example in somaclones derived from a ryegrass × fescue hybrid after two subcultures, the plants appeared phenotypically and cytologically the same as the explant source (Kasperbauer *et al.*, 1979). Plants derived from older cultures had phenotypic changes (e.g. variation in leaf width) and changes in ploidy levels. Not all the morphological changes were correlated to the observed changes in ploidy.

Changes in regenerated plants may not always be obvious. Somaclones may look and grow identically to the parent, but changes in metabolic functions may have occurred. These changes may not be apparent until the plants are evaluated for a specific trait. Screening methods become critical for proper identification of useful variants. For example, bermudagrass somaclones were screened for fall armyworm resistance in laboratory and greenhouse tests (Croughan and Quisenberry, 1989; Croughan *et al.*, 1994a). Several somaclones identified with increased resistance to fall armyworm were evaluated in field tests with the parent cultivar from which they were derived. One somaclone, Brazos-R3 (Croughan *et al.*, 1994b) had equivalent field performance to the cultivar from which it was derived (Eichhorn *et al.*, 1993). This somaclone maintained the same yield, persistence, and forage nutritional quality parameters as the parent. Biochemical tests showed that the somaclone had lower levels of an insect feeding stimulant (Mohamed *et al.*, 1992). This somaclone maintained the positive agronomic traits of the parent cultivar, but had a change in the production of a secondary metabolite that conferred increased resistance to fall armyworm. Development of new cultivars through somaclonal variation have to balance the amount of induced variation while maintaining the important agronomic traits in the parent cultivar.

New molecular methods may be useful in identifying somaclones with genetic variations. For example, PCR methods are being developed in tree species to identify cloned regenerated plants and genetic stability (Isabel *et al.*, 1993). In culture systems that have high rates of regeneration, these methods could be used to screen for variation and reduce the number of somaclones that will be evaluated further. In studies where somaclonal variation needs to be minimized, for example in studying fescue and fungal endophyte interactions (Roylance *et al.*, 1994), molecular methods could be used to identify genetically uniform material.

Conclusions and Prospects for the Future

Clonal variation has been demonstrated many times in cereals and forage crops for both physiological/biochemical and structural/morphological characteristics. These changes are typically undesirable, but the occasional appearance of traits not found in existing populations more than compensates for the large populations of unusable regenerants. Much of the literature simply reports variation following cloning in tissue culture with little attempt to determine the importance of the variation or its heritability. Limited research has been conducted to compare

mutation breeding with chemical mutagens or irradiation to generation of variation through tissue culture. Gavazzi *et al.*, (1987) demonstrated that variation frequencies in tomato for most changed characteristics were higher following somaculture than those caused by the chemical mutagen ethyl methane sulphonate (EMS). Varies-Paterson and Stephens (1990) compared gamma irradiation and somaculture for inducing variation for resistance in tomato to *Clavibacter michiganensis* and demonstrated that somaculture induced this variation at a higher frequency than irradiation.

Clonal variation is most useful for breeding programmes where the desired trait or traits is not known naturally from available germplasm or is not readily available. Examples are salt tolerance, tolerance to mineral toxicities, tolerance to various chemicals including herbicides, tolerance to cold or heat, tolerance to certain pests and pathogens, and variation in useful physiological characteristics. Much of the usefulness of clonal variation is that the variations are generated under *in-vitro* conditions, which allows for *in-vitro* selection among millions of cells for specific characters. Large-scale field screening of plants and progeny is usually limited by available space and labour. Clonal variation in general also has less deleterious effects on mutated plants than chemical mutation or irradiation. Clonal variation also can be combined with the use of chemical mutagens in culture or irradiation of explant materials before plating in tissue culture. In our laboratory we have demonstrated that culturing rice from tissue culture stable varieties on medium containing EMS significantly increased variation in regenerated plants (Xie and Rush, unpublished).

The amount of clonal variation among cultured varieties and lines within a crop varies greatly, with some germplasm highly variable and some tissue culture stable. In rice, the varieties Tetep, Taduken, and Taipei 309 tend to show little variation from somaculture and are considered to be tissue culture stable. Other varieties, such as the US long-grain cultivar Labelle, appear to have some type of variation in nearly all of the derived somaclones. High variability in tissue culture appears to be a heritable trait (Chu and Croughan, 1990). The amount of clonal variation in a crop should be greatly increased by including highly variable germplasm in crosses, by culturing explants from F_1 or F_2 plants from these crosses, and by including chemical mutagens in the tissue culture medium or by irradiating explants before plating.

In our research with rice, we have observed more than 30 types of variation from somaculture. Two elite lines with a high level of partial resistance to sheath blight were obtained from a somaclone from the sheath blight (*R. solani*)-susceptible cultivar Labelle. The high level of partial resistance in the elite lines LSBR-5 and LSBR-33 was demonstrated to be controlled by a recessive gene. This type of sheath blight resistance has not been found naturally in rice. These lines are being used extensively in the rice breeding programme at Louisiana State University to develop sheath blight-resistant varieties. Thousands of progeny rows from crosses with the elite line LSBR-5 and susceptible commercial varieties have been field tested through the F_8 or F_9 generation. The resistance in these lines was stable. According to the literature many rice varieties have been developed

through the use of tissue culture techniques and with useful clonal variations. The very early-maturing, long-grain rice variety Texmont was developed at the Texas A&M University Agricultural Research and Extension Center at Beaumont, Texas using anther culture of the cross RU8303116/Lemont to quickly develop homozygosity in lines derived from the F_1 plant (Bollich *et al.*, 1993). In China, the *japonica* rice varieties Zhonghua No. 6, 8, 9, 10, 11, and 12, developed through anther culture, were planted over an area of 700 000 ha in 1996 with an average yield of 7.5 t/ha (Xu and Chen, 1996). By 1989, more than 100 varieties and promising breeding lines had been released in China after development through anther culture (Gao, 1989). Many of these germplasms show clonal variation.

Tissue culture is an important tool for transformation in crop plants. In general, clonal variation is a disadvantage in transformation research where we are attempting to transform successful commercial varieties with a single trait without changing their other characteristics. However, it is possible that desirable clonal variation may actually improve a transformed cultivar (Linscombe *et al.*, 1993).

In the future, the use of anther culture of F_1 hybrids in rice will continue to be utilized as a tool in the breeding programme. The combination of increased variation and fixing the variation by induction of homozygosity in doubled haploids makes anther culture a valuable breeding tool in crops such as rice where this technique is straightforward and relatively simple. The use of *in-vitro* selection, especially in protoplast culture where single cell derivation of plants is assured, should continue to be a useful technique for inducing difficult traits that are not naturally available in the crop germplasm. Combining *in-vitro* selection with chemical or irradiation-derived mutations should ensure sufficient variation that 'new' traits can be developed. As transformation technology, especially particle bombardment of cells, is made more consistent and large numbers of transformed plants can be generated, clonal variation may become a useful adjunct to transformation in crop development.

Clonal variation should move from the exploratory stages to routine use in future crop breeding programmes. Research on the stability of clonal variation and *in-vitro* selection techniques are pointing the way to future development of new or rare variation.

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4. Genetic Fidelity of Plants Regenerated from Somatic Embryos of Cereals

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Introduction

In-vitro somatic cell and tissue culture technologies have been developed to assist plant breeding. Since the first report on immature embryo culture of maize (Green and Phillips, 1975), extensive documented reports in a whole range of species (Larkin and Scowcroft, 1981; Evans *et al.*, 1984; Maddock and Semple, 1986; Lee and Phillips, 1988; Brown, 1991; Karp, 1991, 1995) have shown undesirable genetic and cytogenetic variation between regenerated plants and the starting material. However, some papers gave the details on the production of agriculturally useful *in-vitro* selected traits of agronomic values. Regenerated plants varied for a wide range of agro-morphological characters. The variability associated with cell and tissue culture has been termed 'somaclonal variation' by Larkin and Scowcroft (1981), which is defined as 'the variation displayed amongst plants derived from any form of cell culture'.

Not all somatic tissue cultures are inherently variable. The idea that variability is inherent to the cell or tissue culture process requires a survey of factors associated with variability (Karp, 1995), such as the degree of departure from organized growth, the genotype (Orton, 1980; Ryan *et al.*, 1987; Carver and Johnson, 1989; Mohmand and Nabors, 1990), the growth regulators used, and the tissue source (Morere-Le Paven *et al.*, 1992). Moreover, ploidy level of the starting material and time spent in tissue culture seem to be of major importance. Phenotypic variation and sterility increased as ploidy and time in culture increased (Cummings *et al.*, 1976; Armstrong *et al.*, 1983; Lee and Phillips, 1987b; De Buyser *et al.*, 1988; Henry *et al.*, 1996).

This chapter gives detail on the present status of understanding the lack of genetic fidelity of regenerated plants, and developments on the analysis of genetic variability derived from *in-vitro* somatic cell and tissue cultures. Moreover, some insights into the use of this variability for cereal crop improvement is also discussed.

The Spectrum of Changes Associated with Somaclonal Variation

The occurrence of genetic variability in tissue culture and regenerated plants has been described in a wide range of species (Evans *et al.*, 1984; Karp, 1995).

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Table 1. Frequency of chromosome number abnormalities among progeny of the seed grown aneuploid DT7DL Chinese Spring wheat versus that in short- and long-term culture regenerants produced by somatic embryogenesis

	Seed stock	Regenerants from short-term culture	Regenerants from long-term culture
Frequency	76/103 (73.8%)[a]	35/103 (33.9%)[b]	24/32 (75%)[a]

[a, b, c]Entries within lines followed by different letters are significantly different at the 0.05 level.

Somaclonal variation was observed in cereal plants derived from different explant sources including immature zygotic embryos. A part of the variability observed among regenerants pre-exists in the cells and tissues of the explant used for culture initiation (D'Amato, 1978; Breiman *et al.*, 1989). The comparison of somaclones with their parental lines can reveal that presumed changes in the regenerated plants originate from uncontrolled cross-pollination or admixture in the source plants (Metakowsky *et al.*, 1987), giving rise to heterozygosity in the starting material. Most of the abnormal aneuploid karyotypes in wheat regenerants, derived from short-term cultures (Table 1), arose from unbalanced chromosome number in the starting material, the immature embryos (Karp and Maddock, 1984; Maddock and Semple, 1986; Henry *et al.*, 1996). However, the presence of various types of genetic variation in regenerated plants indicates that there is no single source of origin of variation; indeed several contributing processes are involved.

Cytoplasmic Changes

The presence of three genomes, chloroplast, mitochondrial and nuclear inside the plant cells, makes it difficult to analyse somaclonal variation, mostly due to the lack of knowledge of the precise relationship between these genomes.

Chloroplast DNA loss

Albinism, the most frequent and noticeable 'gametoclonal variation', is a serious problem encountered in *Poaceae* anther culture. The chloroplast genome appears to be more stable during somatic tissue culture; nevertheless changes in chloroplast DNA and the presence of albinism have also been detected in somatic cell and tissue culture-derived cereal plants (Jähne *et al.*, 1991; Shimron-Abardanel and Breiman, 1991). Our study (Aubry, unpublished results) has shown that both deletions and numerous rearrangements occur in the chloroplast genome organization of wheat albino plants, regenerated from microspore-derived embryos cultured *in vitro* for 4–14 months. For most of these plants, the chloroplast genome is a heterogeneous population of small linear and circular molecules. This hetero-

plasmy could be amplified during tissue culture. Rice somatic embryogenesis results also indicated that long-term culture causes deletions in the plastid genome (Kawata *et al.*, 1995).

Mitochondrial DNA Reorganizations

Somatic embryogenesis results in various cereal species have revealed many conformational and molecular changes in mitochondrial DNA (mtDNA). Mutations (Gu *et al.*, 1994) and deletions (Gengenbach, 1988; Wintz, 1994) in the mitochondrial genome have been described. The differences in the nature of mtDNA variation were observed in plants regenerated from different explants (Morere-Le Paven *et al.*, 1992). Regenerated plants from maize, wheat, rice, triticale, sorghum and sugarcane may have altered mtDNA restriction fragment patterns (Table 2). After short-term cultures, wheat plants regenerated from somatic embryos showed similarity in mtDNA organization to the parental cultivar (Hartmann *et al.*, 1989). Conversely, the mitochondrial genome of Chinese Spring wheat undergoes considerable structural changes and restructuring as a result of long-term *in-vitro* somatic embryogenesis. The process is controlled by nuclear genes (Small *et al.*, 1988; Hartmann *et al.*, 1992), and it has been demonstrated that the *CHM* gene from *Arabidopsis* encodes a protein involved in the control of mtDNA reorganization events (Martinez-Zapater *et al.*, 1992). The new mtDNA subgenomic configurations of the regenerants are inherited via the maternal parent (Gengenbach, 1988; Morere-Le Paven *et al.*, 1994). The differences observed in plants regenerated from the same explant reveal that the new mtDNA organizations or mutants survive heteroplasmically in the plant (Gu *et al.*, 1994; Wintz, 1994). Strengthened by *in-vitro* culture, homologous recombination of the

Table 2. Mitochondrial DNA variations and culture duration in plants regenerated after somatic embryogenesis

Species	Reference
Maize	Bretell *et al.*, 1980; Gengenbach *et al.*, 1981 LT; Gegenbach, 1988 LT; Chourey and Kemble, 1982; Kemble *et al.*, 1982 (6 months); Umbeck and Gengenbach, 1983 (6 months); Fauron *et al.*, 1987, 1990, 1992 (6 months); Small *et al.*, 1988; Gu *et al.*, 1994, LT; Wintz, 1994 LT
Wheat	De Buyser *et al.*, 1988 ST–LT; Rode *et al.*, 1988 LT; Hartmann *et al.*, 1989 ST–LT; Morere-Le paven *et al.*, 1992; and 1994 LT
Triticale	Weigel *et al.*, 1995 (22 weeks *in vitro*); Schmidt *et al.*, 1996 (22 weeks *in vitro*)
Rice	Fukuoka *et al.*, 1994 ST
Sorghum	Elkonin *et al.*, 1994
Saccharum	Chowdhury and Vasil, 1993 LT

ST, LT: respectively short-term (at the most 4 months) and long-term (at least 14 months) tissue culture.

mtDNA and selective amplification can modify the organization of the mitochondrial genome (Schmidt *et al.*, 1996).

All these examples suggest that the mitochondria is highly vulnerable to DNA alterations during long-term somatic embryogenesis. Moreover, the occurrence of a normal phenotype does not guarantee that mtDNA damage has not occurred.

Nuclear Changes

The nuclear genome is much more complex than the organelle DNA, and this makes it more difficult to determine the genetic changes in response to somatic embryogenesis (Brown, 1991).

Cytological Changes

Much is already known about *in-vitro*-induced chromosomal changes. Cytological variations were evident for all tested species (Karp and Maddock, 1984; Karp *et al.*, 1987; Lee and Phillips, 1988). These abnormalities ranged from gross changes such as chromosome number variation (Orton, 1980; Karp *et al.*, 1987) to changes such as chromosome structure (Gould, 1982; McCoy *et al.*, 1982, Karp and Maddock, 1984). Delays in the separation of sister chromatids, chromosome bridges and chromosomal fragments were demonstrated to occur extensively in embryogenic cultures of maize (Fluminhan and Kameya, 1996). Cytological variations were sometimes associated with reduced fertility and altered genetic ratios in the progeny of self-fertilized polyploid plants (Cummings *et al.*, 1996). Nevertheless, minor changes have limited impact on the fitness of polyploid species. As a general rule, polyploid species such as oat and wheat have a high tolerance for chromosomal imbalance when compared with diploids (barley, rye). The chromosome numbers of diploids (if any) are much more stable than those of polyploids. Diploids are vulnerable to chromosomal variation, and do not survive with extensive chromosomal changes. As early as 1980, Orton indicated that chromosomal stability is under genetic control. The type of explant also affects cytogenetic stability (Gonzales *et al.*, 1996).

Even short-term somatic embryogenesis promotes limited chromosome instability in wheat (Henry *et al.*, 1996). Extensive experimental results make unlikely the suggestion that the frequency of abnormal chromosome complements is reduced in wheat somatic-embryo-derived plants (Henry *et al.*, 1996). Chromosome aberrations that occur in cultured cells tend to accumulate with time. Thus, increased duration of *in-vitro* culture, past 90–120 days, has been associated with increased cytogenetic variation in regenerated plants of maize (Lee and Phillips, 1987a; Fluminhan and Kameya, 1996), oat (McCoy *et al.*, 1982), wheat (De Buyser *et al.*, 1988; Henry *et al.*, 1996) (see Table 3). Our experiments demonstrated that most of the regenerants were self-sterile, when originated from wheat somatic embryogenic cultures of 15 months or older. However, a few plants were partially female fertile; these were regenerated 3 years after the initiation of somatic embryogenesis.

Table 3. Frequency of chromosome number abnormalities in Chinese Spring wheat regenerants from short-term and long-term somatic embryogenesis compared to the seed-stock control

	Seed stock	Regenerants from short-term culture	Regenerants from long-term culture
Frequency of abnormal chromosome number	0/254 (0%)[a]	13/369 (3.5%)[b]	68/86 (79.1%)[c]

[a, b, c]Entries within lines followed by different letters are significantly different at the 0.05 level.

Sister chromatid exchanges (SCE) are more sensitive cytogenetic indicators of DNA damage than are changes in chromosome number (Pijnacker and Ferwerda, 1994). SCE induction is strongly enhanced by 2,4-dichlorophenoxyacetic acid (Murata, 1989). The mitotic recombination process could also account for some of the variation (Armstrong *et al.*, 1983; Lapitan *et al.*, 1984). Somatic mitotic genomic rearrangements involving a homologous recombination process have been observed (Das *et al.*, 1990). Meiotic behaviour studies in regenerants showed a higher frequency of chromosome pairing as compared with the starting material; consequently, altered recombination frequencies could be seen by passage through tissue culture (Li and Dong, 1994).

It has been postulated that a high level of heterochromatin results in enhanced chromosome abnormalities. By using ditelosomic somatic embryogenesis, our results demonstrated that the generation of chromosomal abnormalities was equally affected by A, B and D wheat genomes, indicating that there was no clear relationship between heterochromatin level (highest in the B genome) and chromosomal abnormalities (Henry *et al.*, 1996). These results are in contradiction to the theory of late replication of heterochromatin as a potential source of chromosome aberrations induced by tissue culture (Lee and Phillips, 1988). Nevertheless, it seems that primary breakage often occurs inside knobs or at junction regions between the euchromatin and the heterochromatin (Fluminhan and Kameya, 1996).

The regeneration frequencies were identical, 45.7% and 46.3%, respectively, after short-term (4 months) and long-term somatic embryogenesis, whereas the normal chromosome complements were highly significantly different in regenerants, 88.9% and 20.5%, respectively (Henry *et al.*, 1996). These results clearly show the lack of relationship between chromosomal abnormalities and regeneration frequencies, at least during wheat somatic embryogenesis. The frequency of abnormal chromosome complements in regenerated plants is comparative to the frequency of abnormal chromosome numbers in the cells during somatic embryogenesis (Henry *et al.*, 1996). This makes unlikely the hypothesis of a selection in favour of regeneration from cytogenetically normal cells.

The occurrences of chromosomal breakage (McCoy *et al.*, 1982; Singh, 1986) and non-disjunction (Henry *et al.*, 1996) as major cytogenetic phenomena leading

to mitotic abnormalities are well documented in wheat and maize. Chromosome breakage, a non-random phenomenon (Singh, 1986), has been demonstrated in maize to trigger activation of transposable elements. In *in-vitro* culture, stress seems to be sufficient to induce several abnormalities. Heat shock or high salt concentrations have been found to enhance recombination under certain conditions (Puchta and Hohn, 1996). The influence of culture medium components, particularly 2,4-D growth hormone (Murata, 1989), on chromosome instability has been described (Pijnacker and Ferwerda, 1994). Another important biological consequence of recombination is that the genomic change in somatic cells resulting from environmental stress can, in principle, be transferred to the next generation (Puchta and Hohn, 1996). Several mechanisms could be involved in the origin of structural changes, which might not be necessarily limited to the *in-vitro* conditions. Some forms of *in vitro*-induced variation might result from mechanisms that are also operative in *in-vivo* somatic tissues.

Many genomic changes could go undetected even though the phenotype is affected. Unfortunately, there is limited information available both on banding analysis (Gould, 1982; Davies *et al.*, 1986) and the meiotic behaviour of regenerated plants except for wheat (Whelan, 1990), rye (Puolimatka and Karp, 1993) and maize (Lee and Phillips, 1987b). In these cases, extensive studies showed karyotypic changes by meiotic analysis of somatic embryo plants.

Polyploidy, aneuploidy and chromosomal rearrangements are discussed as the main causes for somaclonal variation in cereals. The possible origins of chromosome rearrangements in tissue culture were presented by Lee and Phillips (1988). In several instances, however, phenotypic variation cannot be attributed to cytologically observable changes, suggesting that the changes occur at the molecular level (Brettel *et al.*, 1986a; Brown, 1989).

Mutations

Somaclonal variation often shows Mendelian inheritance for a single factor, and thus appears to involve alterations in nuclear genes. For example, most maize variants were inherited as single-gene recessives (Lee and Phillips, 1987b). The occurrence of induced simply inherited traits is common in cereal somatic embryogenesis cultures. Single base-pair changes could explain a simple Mendelian inheritance in several somaclones (Brettell *et al.*, 1986a). Recent results have demonstrated that some of the tissue culture-induced mutations may be due to the activation of retrotransposons (Hirochika *et al.*, 1996).

Modification in the Copy Number of Repeated Sequences

Repetitive DNA is the most liable DNA to alter in response to stresses. The induction of somatic embryogenesis in plant tissues may induce changes in the copy number of sequences inside the nuclear genomes of rice (Kikuchi *et al.*, 1987; Zheng *et al.*, 1987; Brown *et al.* 1990), maize (Brown *et al.*, 1991) and wheat

(Lapitan *et al.*, 1988). A marked reduction in the rDNA unit number was observed in triticale (Brettel *et al.*, 1986b).

Changes in DNA Methylation Patterns

In somatic embryo derived plants, several sequences underwent significant alterations in DNA methylation status, e.g. in maize (Brown, 1989) and rice (Zheng *et al.*, 1987; Müller *et al.*, 1990). Furthermore, alterations in DNA methylation are widespread in cereals, including triticale (Brown, 1989). Increase or decrease in methylation in response to the *in-vitro* stress has been observed. Such changes were stably inherited through the germline (Brown, 1989; Brettell and Dennis, 1991; Kaeppler and Phillips, 1993). In eukaryotes, epigenetic processes (hereditary modifications of gene expression without change in DNA nucleotide sequence) are often accompanied by changes in chromatin configurations that prevent access to the genes of transcription factors. Such changes are frequently associated with the methylation of cytosine in DNA (Fauvarque and Rossignol, 1996).

Recent evidence implicates cytosine methylation in plant epigenetic phenomena such as gene silencing or inactivation of transposable elements (Ronemus *et al.*, 1996). A reduced methylation of cytosine residues may also be associated with activation of the transposable element *Ac* (Brettell and Dennis, 1991). It is now well documented that methylation is associated with DNA recombination. Loss is methylation, both in repetitive sequence and single-copy-genes, has developmental effects (Ronemus *et al.*, 1996).

Paramutation, the directed heritable alteration of one gene following exposure to another allele in a heterozygote plant, is a mechanism susceptible to induce gene silencing in plants (Martinsen, 1996). In plants where few, if any, genes are subjected to gametic imprinting, no protective mechanisms would exist to prevent the occurrence of methylation transfer as a consequence of interactions (Colot *et al.*, 1996). The transfer of methylation between alleles represents a plausible epigenetic mutational mechanism to explain paramutations in plants (Colot *et al.*, 1996). Moreover, methylation transfer and recombination are mechanistically related (Colot *et al.*, 1996).

Activation of Transposable Elements

Genotypes carrying mobile transposable elements were expected to be more unstable *in vitro*. The activation of transposable elements is considered a possible outcome of DNA methylation alterations in culture. The first report demonstrating that transposition is also an important approach to cause gene mutation in tissue culture was obtained in rice (Hirochika *et al.*, 1996). Although, the activation of transposable elements is largely confined to maize, it also seems to play a role in the production of tissue culture-induced variation. Evidence for more or less altered transposition as a result of somatic embryogenesis has been reported for Activator (*Ac*) sequences (Peschke *et al.*, 1987, 1991; Brettell and Dennis, 1991),

Suppressor–mutator (*Spm*) system (Peschke and Phillips, 1991) and Mutator (*Mu*) sequences (James and Stadler, 1989; Planckaert and Walbot, 1989). Transposable element activity in regenerants indicated that tissue culture-derived variability may result from insertion and/or excision of transposable elements (Peschke *et al.*, 1987). In some instances, altered methylation patterns were produced as a result of *Ac* activity (Peschke *et al.*, 1991).

A recent report indicated that three of the rice retrotransposons were activated under *in-vitro*-stress conditions (Hirochika *et al.*, 1996), and the copy number of *Tos 17* increased with prolonged tissue culture. The transcript of *Tos 17* was only detected under *in-vitro* conditions (Hirochika *et al.*, 1996). Moreover, the results demonstrated that the activation of *Tos 17* causes tissue culture-induced mutations (Hirochika *et al.*, 1996).

Restriction Fragment Length Polymorphism and Randomly Amplified Polymorphic DNA Studies

An accurate determination of changes in a particular gene sequence can be obtained by RFLP and RAPD analysis. RAPD analysis appears to be an efficient tool to monitor genomic changes resulting from tissue culture. Peter Brown examined the molecular basis for somaclonal variation in several species (Brown *et al.*, 1990, 1991, 1993; Müller *et al.*, 1990; Brown, 1991). A large number of RFLPs were recorded in regenerated maize (Brown, 1989; Brown *et al.*, 1991), rice (Müller *et al.*, 1990; Bao *et al.*, 1996) and wheat (Brown *et al.*, 1993) plants. In rice, doubling the time of *in-vitro* culture resulted in a quadrupling of detected DNA polymorphisms in the regenerants (Müller *et al.*, 1990). The length of *in-vitro* culture affected the stability of sequences such as the actin gene (Brown *et al.*, 1990). Nevertheless, variation is not associated with any particular region of nuclear DNA (Brown, 1991). Experimental results suggested that '*in-vitro* stress-induced DNA polymorphisms were then affected by methylation' (Brown *et al.*, 1990). This suggests that DNA methylation may represent a secondary process required to maintain an epigenetic state once it is established (Ronemus *et al.*, 1996).

Many polymorphisms are heterozygous, recessive, and as such do not appear until selfing is done. However, there are different molecular mechanisms which operate to induce variation, and probably a number of processes operate simultaneously. This leads to variation for traits under simple genetic control and also for quantitatively inherited characters.

Interest for Cereal Improvement

In most cases, extensive agronomic trials indicated neither beneficial nor detrimental influence during tissue culture (Mohmand and Nabors, 1990; Morrish *et al.*, 1990; Qureshi *et al.*, 1992; Baillie *et al.*, 1992). The significant variation was found in nearly all the characters evaluated in wheat regenerants (Ryan *et al.*,

1987). However, studies on somaclonal variation in cereals produced contrasting reports, but promising results have been obtained in oat (Dahleen *et al.*, 1991), rye (Bebeli *et al.*, 1993a), pearl millet (Nagarathna *et al.*, 1993), finger millet (Pius *et al.*, 1994) and triticale (Bebeli *et al.*, 1993b). Variation increased with the duration of culture (Symmilides *et al.*, 1995), and most plants regenerated from wheat protoplasts were sterile (Henry and De Buyser, 1997). A limitation to the interest in somaclonal variation was mentioned as early as 1983 (Beckert *et al.*, 1983) in maize, where the variation in regenerants was of limited extent when compared with the genetic variability within the species.

Somatic embryogenesis-derived genetic variability and its impact on cereal crop improvement strategies has been reviewed (Evans and Sharp, 1986; Karp, 1991, 1995). Despite the wide range of heritable variability observed in regenerants, limited attempts have been made to isolate variants for integration into breeding programmes (Maddock and Semple, 1986; Qureshi *et al.*, 1992). Improvement of genotypes by somaclonal variation, has produced very limited results.

There are indications of some success in the use of cereal tissue culture-induced variability with release of few cultivars. The early-maturing high-yielding wheat variety Zdravko was obtained through somaclonal variation in Bulgaria, and registered in 1994 (P.H. Boyadjiev, personal communication). Somaclonal variation also succeeded in producing new cultivars of *Sorghum* resistant to insects (Duncan *et al.*, 1991a) or acid soils (Duncan *et al.*, 1991b, 1992). To date, 'Hatsuyume', a protoplast-derived rice cultivar, has also been released (Ogura and Shimamoto, 1991). Somaclonal variation is probably a more promising approach for improving species having a narrow genetic base, limited breeding systems, without normal sexual reproduction or are vegetatively propagated.

Currently, the top priority of plant breeders is to breed cereal crops resistant to diseases, herbicides, etc. Thus, the possible application of somaclonal variation in cereal species is *in-vitro* selection in the presence of a selective agent such as toxin, herbicide, amino acid analogues or other selective coumpounds (salt, metal ions, etc.). Barley and wheat plants regenerated from calli, which survived the selection pressure of *Helminthosporium sativum* toxin, were more tolerant to disease (Chawla and Wenzel, 1987). Toxin-insensitive plants produced progeny resistant to *Helminthosporium victoriae* (Rines and Luke, 1985).

Somaclonal variation may be a useful tool when mitotic recombination or mtDNA reorganization occurs during tissue culture. Up till now, gene introgression from one species to another has used meiotic interchanges and translocations between chromosome arms or chromosome fragments from related species. Such an approach has been used for conventional cereal improvement for the past 100 years. This has increased interest in the use of mitotic genomic rearrangements for alien introgression by *in-vitro* culture. Translocations between wheat and rye chromosomes have been observed in regenerants of wheat × rye hybrids (Armstrong *et al.*, 1983; Lapitan *et al.*, 1984). Moreover, barley yellow dwarf virus resistance has been transferred to *Triticum aestivum* from *Thinopyrum intermedium* in several recombinant lines induced by cell culture (Banks *et al.*, 1995).

Long-term somatic embryogenesis from cereal immature embryos may also be useful in deriving new mtDNA organizations (Hartmann *et al.*, 1989; Morere-Le Paven *et al.*, 1992; Wintz, 1994), which is otherwise extremely difficult to achieve by classical mutagenic techniques.

The possible uses of tissue culture-induced retrotransposition were recently suggested by Hirochika *et al.* (1996). Tissue culture-induced activation of rice retrotransposons may be a useful tool for gene tagging by insertional mutagenesis and functional analysis of genes, since it induces mutations at high frequency with an original copy number from 1 to 4 (Hirochika *et al.*, 1996).

Some requirements must be met before somaclonal variation can be efficiently used for plant breeding. These are:

1. induction of a wide range of variability;
2. selected characters must be heritable and stable;
3. somaclonal variation will be of great interest when *in-vitro* selected material will correlate with the field phenotypes;
4. controlled production of desired somaclones.

Conclusions

Somaclonal variation has been described as ubiquitous (Karp, 1991). A wide range of genetic changes arose via *in-vitro* plant cell and tissue cultures, and among cereal somatic embryo derived plants. Increasing evidence has indicated that cereal genomes are more responsive to the *in-vitro* stress than hitherto suspected. In the past 10 years, there has been tremendous progress in understanding the origin of somatic embryogenesis-derived variation, especially as a result of major strides in molecular studies. The major cause of somaclonal variation is the modification of DNA repair mechanisms during tissue culture. As the causes of variation become more clearly understood, the prospects of gaining control over stability or variation during *in-vitro* culture become brighter and brighter.

Both Mendelian and non-Mendelian (epigenetic or cytoplasmic) inheritance patterns have been observed in somaclones. In some cases, epigenetic control was documented: methylation produces changes in gene activity that can be transmitted to the next generation, and may revert back to normal or control (Brown, 1989; Kaeppler and Phillips, 1993). Homozygous variation in rice somaclones, however, suggested that homozygous changes in DNA methylation patterns were generated by the *in-vitro* process (Xie *et al.*, 1995). The progeny analysis of regenerated plants demonstrated that a part of the variations was heritable.

The tissue culture-induced changes can be identified by protein marker analysis. The expression of proteins during the somatic embryogenesis process needs to be investigated using high-resolution electrophoresis coupled with immunological studies (Nato *et al.*, 1997). This is an efficient approach to monitor gene expression changes. For example, the rapid synthesis of signalling elements (G proteins, nucleoside diphosphate kinase, mitogen-activated protein kinases, etc.) is a highly

conserved cellular response, redistributing gene transcription and mRNA translation, and thus playing a fundamental role in the regulation of development in eukaryotes. The signalling cascades a large array of upstream events (hormones, stress, starvation, etc.) to a wide range of downstream physiological events. We must assume that the study of plant signalling elements related to cell proliferation and differentiation is of major interest for understanding the *in-vitro*-induced variations.

As a general rule, abnormalities appearing in plants, regenerated from short-term somatic embryogenic cultures, can mostly be related to abnormalities in the initial line. On the other hand, the longer that somatic embryogenesis continues before plant regeneration, the higher the frequency of abnormalities in regenerated plants and their progenies. Cytogenetic and molecular results have shown a limited amount of variation in short-term somatic embryogeneic cultures. On the contrary, extensive variations were revealed in long-term cultures (Chowdhury and Vasil, 1993; Chowdhury *et al.*, 1994). This suggests that the genetic fidelity of cereal somatic embryo plants occurs mostly in short-term cultures using selected original explant and medium. The genetic fidelity is also dependent on the genotype of the explant (Morrish *et al.*, 1990). Publications from Vasil's laboratory indicate that the somatic embryogenesis approach is the best way to maintain the genetic fidelity of regenerated cereal plants (Chowdhury and Vasil, 1993; Chowdhury *et al.*, 1994; Morrish *et al.*, 1990). We have carried out detailed cytogenetic and molecular studies, some very substantial in nature, showing that genetic changes occur through long-term *in-vitro* cereal somatic embryogenesis, a process which does not ensure genetic fidelity. This is not surprising in oat and wheat, for example, and to a lesser extent in maize, which is not a true diploid (Moore *et al.*, 1995). It is well known in the polyploid species that even the zygotic embryos might possess chromosomal structure different from the euploid state: ditelosomic, nullisomic–tetrasomic and various different aneuploids are viable in plants, and thereby are viable as embryos.

The discovery of variation in somatic embryo derived plants led to a flurry of interest in the 1980s. The potential of somaclonal variation to act as a new useful source of variability has been discussed extensively for cereal breeding. It seems, that there is no longer interest in *in-vitro*-induced variation. The current thinking of researchers is that somaclonal variation has limited advantages and significant disadvantages. Therefore, the expected contribution of somaclonal variation has not yet been fully realized in cereal breeding. Nevertheless, cereal somatic embryo derived plants may furnish valuable, and useful germplasm for breeding purposes. Some new avenues have already been seen. The major problem of somaclonal variation is that it is random and unpredictable, and thus is not strictly reproducible. It will be necessary to improve our understanding of the ubiquitous causes of the heritable changes in order to predict genetic fidelity of plants regenerated from somatic embryogenesis in cereals.

Of the various currently used *in-vitro* biotechnologies in cereals, somatic embryogenesis has had limited application in genetics and breeding. Recent new gene transfer technologies (protoplast culture, particle gun, *Agrobacterium*-based

methods) could be considered to use genetic engineering for cereal improvement. Fortunately, genetic engineering of cereal crops mostly relies on somatic embryogenesis. However, *in-vitro*-induced variability remains a major limitation to the use of transformation procedures in cereals, due to genomic changes in transgenic plants (Bao *et al.*, 1996) regenerated from long-term cultures. Therefore, the use of immature explants and short-term somatic embryogenesis for particle gun technology increases the range of transformable genotypes and reduces the incidence of somaclonal variation.

The maintenance of genetic fidelity in regenerated plants is of paramount importance for cereal biotechnologists. We suggest the use of short-term somatic embryogenesis from selected explants of particular genotypes. Extensive chromosomal variation can occur in wheat somatic embryo plants, that express *Em* genes (Corre *et al.*, 1996). This suggests that plant regeneration through somatic embryogenesis in polyploid species does not ensure normality (Henry *et al.*, 1996). The situation in hexaploid wheat is not representative for diploids, in which changes in chromosome number and structure often have an important impact on the fitness of plants.

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5. Somaclonal Variation in Improving Ornamental Plants

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Introduction

Some 40 years ago Braun (1959) reported that *in-vitro*-cultured cell clones may display genetic alterations. Genetic differences have also been found in and among plants originated by adventitious regeneration from somatic cells (Larkin and Scowcroft, 1981) and in their progenies (Edallo *et al.*, 1981; Evans and Sharp, 1983; Buiatti *et al.*, 1985). Genetic variation occurring in such tissue-culture-derived plants has been defined as 'somaclonal variation' by Larkin and Scowcroft (1981).

The discovery of the high extent of genetic variation in tissue-cultured plants coincides with other findings on genetic instability. In contrast with animals, plants often show a high degree of somatic genetic heterogeneity (Buiatti, 1988, 1989). This has been demonstrated in elegant cytologic studies (D'Amato, 1989) in which the ubiquitous presence of endopolyploidy and other chromosomal and genomic mutations in somatic tissues was observed concomitant with differentiation. Later work showed the presence of transient and multiple permanent changes such as DNA content per cell or differential DNA replication during differentiation and dedifferentiation in several species (Buiatti, 1977; Evans, 1989; Nagl, 1990). Permanent DNA methylation or hypomethylation were prominent factors in the contribution of permanent or transient changes in cells and plant phenotypes (Matzke and Matzke, 1993).

The discovery of 'the jumping genes' by McClintock led to what are known today as 'transposons' (McClintock, 1984). They act as a source of somatic genetic variability in plants and give further support to a picture of plants as genetically heterogeneous cell populations on which ontogenetic and environmental constraints may act as selective factors. This, in fact, had been a part of the tradition of ornamental plant breeders whose major successes in vegetatively propagated plants have often been based on the selection of favourable somatic mutations observed in field populations.

The reports on somaclonal variation raised great enthusiasm among plant breeders and raised high hopes of exploiting such variations either directly or by *in-vitro* selection. The aim of this review is to assess the use of somaclonal variation in ornamental plants.

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Table 1. Variation frequency in some ornamental plants

Species	Frequency (%)	Authors
Saintpaulia	10.0	Khokhar, 1983
Saintpanlia	1.5	Winkelmann and Grunwaldt, 1995
Dianthus	0.8	Buiatti *et al.*, 1986
Dracaena	10.0	Debergh, 1976*
Eustoma	1.3	Griesbach and Semeniuk, 1987
Lilium	0.0	Takayama *et al.*, 1982
Kalanchoe	0.2–23.4	Schwaiger and Horn, 1988
Chrysanthemum	0.0–60.0	Khalid *et al.*, 1989

*See Debergh (1992).

Assessment of the Extent of Somaclonal Variation

Somaclonal variation may result in qualitative changes. Depending on the genotype and the tissue culture conditions, the frequency of qualitative changes varies. Jain (1993a,b) observed no variation in flower colour of *Begonia* and *Saintpaulia* somaclones. *Begonia* somaclones did not produce any albino plants but *Saintpaulia* did produce them (Jain, 1997). Different somaclonal variation frequencies were reported starting from 6–17% in *Lycopersicon esculentum* (Evans and Sharp, 1983; Buiatti *et al.*, 1985), 20–33% in *Zea mays* (Armstrong and Phillips, 1988), 47% in *Nicotiana sylvestris* (Prat, 1983), and 2% in *Nicotiana tabacum* (Dulieu, 1986). Table 1 shows that similar values have also been obtained in ornamental plants, even though variation between species is greater.

In tissue culture-derived plants, variation in quantitative characteristics has been observed. Such variation concerns ornamental plants, e.g., changes in the duration of vase-life or the number of flowers produced per plant. De Klerk *et al.* (1990) and Bouman and de Klerk (1997) used changes of a quantitative character, *viz.* leaf shape, to assess the extent of variation. After chemical mutagenesis, changes had occurred, as shown by an increase of the coefficient of variation of the leaf shape in a population of regenerated plants whereas the mean leaf shape remained unaltered. A large increase of variation was also observed after regeneration from non-organized callus. They discussed the problems of distinguishing true genetic variability from epigenetic variation, possibly with high rates of reversion frequency. Therefore, measurements based on quantitative parameters such as the coefficient of variation, proposed by De Klerk *et al.* (1990), certainly have a practical value but should be confirmed through several generations of vegetative propagation. Jain (1993 a,b) observed a decline in the value of standard deviation for flower size of *Begonia* and *Saintpaulia* from the MV2 to the MV3 generation, indicating a reduction in variability. Table 2 shows stability of selected somaclones of *Begonia* and *Saintpaulia* after micro-propagation for 2 generations. Heritability estimates (Buiatti *et al.*, 1986), although being more reliable on a theoretical basis, are rather laborious. Frequencies of plants with aberrant morphology may also be misleading due to the frequent recovery of normal

Table 2. Evaluation of selected *Begonia* and *Saintpaulia* somaclones in the MV2 and MV3 genetations. (Jain, 1997)

Clone	*Begonia*		*Saintpaulia*	
	MV2	MV3	MV2	MV3
190	+	+		Flower diameter
250	+	+		No. of flowers/plant
275	+	+		No. of flowers/plant
UV 186	Flower diameter		+	+
UV 28	No. of flowers/plant		+	+
UV 150	No. of flowers/plant		+	+
180	+	+		Flower diameter
200	+	+		Flower morphology
220	+	+		Plant morphology
UV 175	Flower diameter		+	+
UV 200	Flower morphology		+	+
UV 50	Plant morphology		+	+

+, Stable.

Each somaclone was selected in MV1 for a specific character (written after each somaclone) and micropropagated for two generations.

phenotypes while characters such as flower colour may be evaluated only on the basis of solid mutants, due to the effects of diplontic selection on persistence of mutated sectors in subsequent generations (Buiatti *et al.*, 1970).

In all cases, however, variation seems to be highly species-dependent as shown by the data on tobacco (Dulieu, 1986; Bettini *et al.*, unpublished) and on barley (Breiman *et al.*, 1987; Shimron and Breiman, 1991), showing little or no evidence of mutations. The phenotypic spectrum of heritable changes in tissue culture-derived plants is often similar to *in-vivo* recovered mutants, although new phenotypes have been observed in several instances. Dominant mutations have been more frequent in *in-vitro* regenerants and their progenies and such mutations have often been homozygous. Although the overall frequencies are much higher, the nature of changes has closely reflected somatic genetic variability *in vivo*. Thus, aneuploidy and polyploidy are extremely frequent (D'Amato, 1989) in *in-vitro* cultures. Although few aneuploids may be recovered among regenerants, polyploids often tend to persist during ontogenesis and can reach frequencies as high as 75% in potato regenerated plants (Karp, 1991).

Molecular Analysis of Somaclonal Variation

Changes in Electrophoresis Patterns of DNA Fragments

Newly developed molecular methods may be more reliable for documenting somaclonal variation (Henry, 1997). However, different markers measure different

classes of genetic variability and exclude certain regions of the analysed genomes. Another major problem is that these techniques may be insufficiently sensitive (Bouman and de Klerk, 1997).

Restriction fragment length polymorphism (RFLP) analysis reveals variation between the specific restriction sites of a few sequences at a time. This technique is dependent on the natural variation in DNA base sequence, and digestion of DNA which differs in size, or length and can be used as a genetic marker to follow chromosome segments through genetic crosses. Muller *et al.* (1990) examined rice regenerants by RFLP analysis to determine the occurrence and extent of somaclonal variation. DNA polymorphisms were observed both among plants regenerated from different callus cultures and from a single callus. Furthermore, differences in RFLP patterns can arise by two mechanisms: (a) direct point or structural mutations, or (b) a change in methylation pattern. A change in methylation pattern may explain some of the genetic variation among regenerated plants, especially with the elevation of 5-methylcytosine as well as the possible presence of minor 6-methyladenine (Muller *et al.*, 1990). Chowdhury and Vasil (1993) found no DNA variation in sugarcane plants regenerated from callus, cell suspension cultures and protoplasts analysed by RFLPs. In contrast, RFLP analysis has detected somaclonal variation in sugarbeet (Sabir *et al.*, 1992), and oil palm (Jack and Mayes, 1993).

Microsatellites or short sequence repeats (SSR) are repeating di-, tri- or tetranucleotide units often found within regulatory sequences or introns, and may be highly polymorphic within and among related species (Tautz, 1989); hence they may be useful in the characterization of somaclones. Polymerase chain reaction (PCR) amplification of the microsatellites using primers to unique sequences that flank the repeat region results in fragments of specific length depending upon the allele(s) present within a specific organism. Microsatellites can distinguish a large number of alleles but require considerable effort to isolate and characterize because of the prerequisite that DNA sequences containing them must be known. Polymorphic microsatellite loci often have a large number of alleles (Roder *et al.*, 1995). Veilleux *et al.* (1995) established homozygosity of potato clones by SSRs and RAPDs. Wolff et al (1995) used SSR analysis for the identification of chrysanthemum cultivars and found absence of polymorphism among different accessions of the same cultivars, indicating genetic stability.

Random amplified polymorphic DNA (RAPDs) are another type of molecular marker that allows the scanning of the genome for genetic changes (Williams *et al.*, 1991). By RAPD analysis more random screening of larger parts of a genome can be achieved. This analysis is less reliable when the resultant polymorphisms are not carefully tested. RAPDs may represent a part of the genome that is more readily amplified due to its composition (Buiatti and Bogani, 1997). Taylor *et al.* (1995) observed very few RAPD polymorphisms in sugarcane plants regenerated from embryogenic cultures, indicating infrequent gross genetic changes during tissue culture. Heinze and Schmidt (1995) found no gross somaclonal variation in somatic embryos and somatic embryo plants of Norway spruce (*Picea abies*) by RAPD analysis. The genetic fidelity of somaclones can be

monitored by RAPD analysis; however, this technique is still not sufficiently accurate to identify point mutations.

Another marker system, amplified fragment length polymorphism (AFLP), has a great potential to analyse genetic variation. This method involves digestion of genomic DNA with restriction enzymes, ligation of linkers and selective PCR amplification with primers complementary to the linkers but with unique 3′ overhangs (Zabeau and Vos, 1993). By this technique very large numbers of markers can be detected, and this allows identification of relatively low levels of somaclonal variation.

In conclusion, many authors have reported specific changes in DNA by various techniques (RFLP, RAPD), whereas new techniques (AFLP, SSR) still have to be exploited. Changes in RFLP and RAPD patterns occur only very infrequently and cannot be used to predict the extent of variation in a somaclonal population unless a very extensive (and therefore costly) examination has been done.

Gross Changes in DNA

Early reports indicated that DNA amplification (Buiatti, 1977; Nagl, 1990) occurred frequently at the beginning of cell proliferation (Parenti *et al.*, 1973). Such amplification was dependent on culture conditions and necessary for differentiation in *Nicotiana glauca* pith tissue (Durante *et al.*, 1977). Multiple changes in DNA, moreover, can be transmitted to the regenerated plants and their progenies, as shown in flax (Cullis and Cleary, 1986), wheat × rye hybrids (Lapitan *et al.*, 1988), *Nicotiana sylvestris* (De Paepe *et al.*, 1982), *N. tabacum* (Dhillon *et al.*, 1993), *Oryza sativa* (Sun and Zheng, 1990). While circumstantial evidence exists on the correlation between the physiological state at the cellular level and multiple changes in repeated sequences (particularly of r-DNA), no clear data are available on the effect of such phenomena on the phenotype of regenerated plants and their agronomic value. However, this phenomenon may indeed exist, based on the following evidence: a decrease in yield of *N. tabacum* dihaploids with high DNA content (Reed and Wernsmann, 1989); the classical work on the PL cultivar in flax studied *in vitro* and *in vivo* (Durrant, 1962) and molecular evidence (Cullis, 1986).

DNA methylation and hypomethylation processes are known to affect gene expression and may grossly change phenotype. This process frequently occurs *in vitro* and in regenerated plants and their progenies (Muller *et al.*, 1990: Kaeppler and Phillips, 1993a,b). Karp (1991) suggested that changes in methylation may be responsible for high frequencies of heritable variation and of the observed true-to-type fully homozygous progenies of regenerated plants. Hypomethylation, in fact, has been shown to induce gross changes in the phenotype. e.g. dwarfing in rice (Sano *et al.*, 1990). The changes in methylation patterns are also associated with carrot embryogenesis (Vergara *et al.*, 1990) and tissue culture habituation (Bogani *et al.*, 1993). It should be noted that methylation is known to increase the frequencies of CG to AT mutations and, therefore, may be considered one of the reasons for high *in-vitro* mutation frequencies (Phillips *et al.*, 1994). Moreover, expansion of simple sequence repeats (SSRs) occurring through

slippage and mutations deriving from amplification of repeated sequences (RIP) are both newly discovered but relevant sources of permanent genetic variability.

Extrachromosomal variation, although almost limited to mitochondria, is also a common feature of tissue culture and regenerated plants. It may affect agronomically important characters, such as male sterility, resistance to pathogens (Umbeck and Gengenbach, 1983) and regeneration capacity (Hartmann *et al.*, 1989).

Causes of Molecular Changes in DNA

The debate still continues as to the cause(s) of high mutation spectra and frequency rates that are not entirely overlapping with those found in nature. Mutation spectra largely reflect somatic changes and are associated with development. Explants are genetically heterogeneous and cell proliferation *in vitro* is unaffected by selective constraints operating *in vivo*, allowing diplontic selection, i.e. selection among diploid somatic cells, without adherence to ontogenetic 'rules' (Buiatti, 1988, 1989; Gaul, 1961). Such a selection process is influenced by environmental and physiological factors (Buiatti *et al.*, 1970). Buiatti *et al.* (1970) showed differences in mutation frequencies and sector sizes between irradiated gladiolus plants, grown in the open field and in the greenhouse. On the other hand, the lack of ontogenetic constraints may be one of the causes that enhances mutation rates *in vitro*, which has been proven beyond any doubt at the cytological (Bennici *et al.*, 1968) and molecular levels (Brown *et al.*, 1991).

Stress exerted by *in vitro* culture conditions may be an additional factor that increases mutation frequencies through impaired replication and drastic modifications of cell metabolism. McClintock (1984) suggested that the induction of genetic variability or any source of genetic instability during stress may be a prerequisite for adaptation. In general, any source of genetic instability during *in-vitro* culture, with stress as the causal factor, may activate transposable elements (Peschke and Phillips, 1991; Peschke *et al.*, 1991). Recently, the effect of stress has also been observed directly in some cases (Buiatti and Bogani, 1997). For instance, *Actinidia* plants regenerated on media containing osmotic agents (3 ortho-methyl-glucose, *N*-methyl-D-glucamine, NaCl) showed a considerable amount of variation by RAPD analysis when compared with control plants displaying non-polymorphic patterns (Muleo *et al.*, submitted). An increase in polymorphism with osmotic stress was also observed in tomato (Boscherini *et al.*, Kaltzikes and Bebeli, personal communications).

The differences in spectra between *in vivo* and *in vitro* mutations and/or epigenetic modifications may be explained by recalling somatic mutation spectra in explants of differentiated tissues. The increase in somatic mutation frequency rates may be attributed to a series of causes determining what McClintock defined as genomic stress. Both facts, obviously, are interesting for the breeder, who can reasonably expect to recover rare, dominant mutations with a frequency higher than normal. This is of particular relevance for breeders working with ornamental vegetatively propagated crops where diploidy or polyploidy may be an obstacle to the

exploitation of recessive mutations and frequencies of 'bud sports' frequencies are generally low.

The Spectrum of Heritable Changes in Ornamental Somaclones

Many *in-vitro*-grown and regenerated ornamental plants show some sort of somaclonal heritable variation, which may influence plant morphology, leaf morphology, flower colour and shape, leaf variegation etc. (Table 3). Although based on a few studies, modifications in quantitative characters have also been observed, leading to an increase in variability which can later be exploited through selection for desired traits. For instance, Buiatti *et al.* (1986) obtained higher heritability estimates in carnation plants (cv *Corrida*) regenerated from petals than from meristems or from normal cuttings for characters such as plant height, flower number, and flowering data. De Klerk *et al.* (1990) observed higher standard

Table 3. Somaclonal variation observed in regenerated ornamental plants

Species	Plant morphology	Leaf morphology	Flower colour	Flower shape	Leaf variegation
African violet	–	+	+	+	–
Begonia	+	+	+	+	–
Anthurium	+	–	–	–	–
Chrysanthemum	+	+	+	+	–
Carnation	+	–	+	+	–
Coleus	–	–	–	–	
Cordyline–Dracaena–Ficus	–	–	–	–	+
Cyclamen	+	+	+	+	–
Dahlia	+	–	–	–	–
Daylily	+	–	–	–	–
Fuchsia	–	–	+	+	+
Gerberra	+	+	+	–	–
Haworthia	+	+	–	–	–
Kalanchoe	+	+	+	+	–
Kohleria	+	+	+	+	–
Marguerite	+	+	–	–	–
Nicotiana alata	–	–	–	+	–
Orchids	–	–	+	+	–
Pelargonium	+	+	+	+	+
Petunia	–	+	–	+	–
Poinsettia	–	–	–	–	+
Ranunculus	+	–	+	–	–
Rhododendron	+	+	–	+	–
Rose	+	+	–	–	–
Rudbeckia	–	–	–	+	+
Weigela	+	+	–	+	–

+, Present; –, absent.

deviation of the ratio between lengths of leaf ribs in regenerated *Begonia* than in control plants (Jain, 1993a,b). Similarly, an increase in variability for flowering date, plant height, plant width, number of flowers and flower diameter was reported for *Chrysanthemum* (Votruba and Kodyteck, 1988), *Begonia* (Jain, 1993a,b,c), and *Saintapulia* (Jain, 1993a,b,c). Furthermore, quantitative variation was observed in *Rudbeckia* regenerated plants for number of ray florets flower (Khibas, 1995), *Begonia* and *Saintpaulia* (Jain, 1993a,b,c; 1997).

It should be emphasized that ornamental plants, which are often vegetatively propagated, can differ permanently for epigenetic changes, chimera rearrangements and chromosomal variation that would be lost in seed-propagated plants due to strong selection pressure at the meiotic and gametic levels. Thus, carnation plants regenerated from petals tend to 'remember' the physiological phenotype of the initial explant and, therefore, flower very early, almost without vegetative growth (Gimelli *et al.*, 1984). This character was inherited as a single gene (Sparnaaij, personal communication).

Epigenetic variation, due to long-lasting gene differential inactivation, may be the cause of the many instances known in vegetatively propagated plants of semi-permanent reversion to juvenile state. In *Begonia*, especially among plants regenerated from long-term callus cultures, an acquired stable juvenile phenotype has often lasted for more than 3 years but was lost after a second regeneration step from leaf explants in the presence of 5-azacytidine (a hypomethylating agent) (Bouman and De Klerk, 1997). Table 4 shows that chimeral rearrangements are very often observed in regenerated plants, and can readily be exploited to obtain new plant types, particularly when variegation patterns have specific commercial importance in ornamental plants such as *Dracaena*, *Pelargonium* and many others.

Polyploidy and aneuploidy can readily be maintained in vegetatively propagated ornamental plants in subsequent generations (Table 5). Although mutation frequency rates may vary among species, they are generally lower in regenerated plants than in callus. Thus, in *Anthurium*, Geier (1988) observed only two polyploids among 667 plants. Miyazachi and Tashiro (1978) observed 100% true-to-type plants in *Chrysanthemum* from a callus exhibiting almost 70% variation in chromosome number. On the other hand, Ogihara (1990) obtained, from a *Haworthia* stock callus, normal karyotypes (55% cells), hypodiploidy (29% cells) and tetra-hypotetraploidy (12% cells), 71% normal plants, 11% hypodiploid and 17% tetrahypotetraploid plants. Cassells and Morrish (1987) made an interesting observation that a specific abnormal chromosome number mode (17) could become fixed in *Begonia* plants regenerated from callus showing a bimodal distribution (17–24).

Frequency Modulation of Somaclonal Variability

As mentioned earlier, several factors are known to influence mutation frequencies in *in-vitro* and in regenerated plants. The control of the extent of obtained varia-

Table 4. Some examples of chimaera rearrangements in regenerated ornamental plants

Species	Explant (and multiplication system)	Chimaera (frequencies)	Rearrangement	Reference
Carnation	Plantlets	Flower colour Y/R/R	52% N	Silvy and Mitteau, 1986
cv. Jacqueline	(adventitious shoot regeneration)	48% Y/Y		
cv. White Sim	Shoot tip and subapical regeneration	Flower colour W/R 99. 0.5% R/R	5% W/W	Dommergues and Gillot, 1983
Chrysanthemum	Petals–epidermal peels	Pigmented LI	100% pigmented LI	Bush *et al.*, 1976
		non-pigmented LII	100% pigmented LII	
	Multiple shoots of apex	id.	1/3 normal 2/3 pigmented LI/LII	
Cordyline and Dracaena	Micropropagation Callusing	Variegated leaves	100% not true to type	Many authors
Pelargonium spp.	Protoplasts	Variegated	leaves (green, albino, variegated)	Kameya, 1975
Peperomia spp.	Adventitious shoots from leaves	Variegated leaves	> from LIII, < from LII few chimaeras	Bergann and Bergann, 1982

Table 4. Some examples of chimaera rearrangements in regenerated ornamental plants (contd)

Species	Explant (and multi-plication system)	Chimaera (frequencies)	Rearrangement	Reference
Poinsettia cv. Annemie pink	Adventitious shoots from leaves	Bracts colour W/R/R	Annemie R/R/R/ Annemie marble W/W/R Annemie white W/W/W	Preil, 1986
7 Pink cvs	Cell suspension	Bracts colour	5/7 chimaeras (no pink bracts in progeny)	Preil *et al.*, 1983
cv. Anneette hegg	Cell suspension	Different morphology	35% variant plants	Preil and Engelhardt 1982
Rhododendron	Shoot tip-differentiated explants	Variegated leaves LI–LIII	Seven different variegation patterns, reverse of original	Pogany and Lineberger, 1990
Saintpaulia cvs	Leaf explants	Variegated leaf pinwheel flowering	Nearly 100% not true-to-type reverse of original	Lineberger *et al.*, 1993

Yellow, Y; Red, R; Normal, N; W, White.

Table 5. Species in which chromosomal variations have been observed in regenerated plants

Polyploidy	Aneuploidy	Polyploidy and aneuploidy
African violet	*Chrysanthemum*	*Begonia*
Anigozanthos	*Rudbeckia*	*Haworthia*
Anthurium		*Pelargonium*
Cyclamen		*Saintpaulia*
Day lily		
Dendrobium		
Fuchsia		
Kalanchoe		
Nicotiana alata		
Petunia		
Pointsettia		
Rudbeckia		

tion may have relevance for ornamental plant breeders wanting to reach an equilibrium between the negative effects of random variation in propagation and the positive induction of useful mutants. A series of experiments were conducted with the aim of evaluating the impact of specific modulating factors. While interspecific variation is a common experience (Table 1), intraspecific differences between cultivars and clones have been observed in *ad-hoc* analysis in *Pelargonium* (Skirvin and Janick, 1976a), *Kalanchoe* (Schwaiger and Horn, 1988), *Chrysanthemum* (Khalid *et al.*, 1989), and *Begonia* (Westerhof *et al.*, 1984) (Table 6). Khalid *et al.*, (1989) found stable chromosome numbers in some *Chrysanthemum* cultivars, whereas others varied up to 20%. As mentioned above, early induction of flowering in carnation occurred in all 'Rubino' plants regenerated from petals, was never observed for 12 other cultivars, and variation ranged from 1% to 97% in four additional genotypes.

A few examples of different mutation frequencies in cells and plants regenerated from different initial explants are reported in Table 7. Buiatti *et al.* (1986) showed lower variation in carnation plants derived from meristems than in those obtained from callus. These observations were supported in other studies on *Chrysanthemum* (Khalid *et al.*, 1989; Miyazchi and Tashiro, 1977, 1978), in which an increase in flower colour mutations in plants regenerated from stem segments, and to a lesser extent from capitula, when compared with those derived from shoot tips.

Data obtained in *Kalanchoe* (Schwaiger and Horn, 1988), daisies (Gimelli *et al.*, 1993), and *Pelargonium* confirmed the presence of higher variation in plants derived from the differentiated rather than from the dividing tissues. This fact probably reflects somatic variation observed *in vivo* as a result of differentiation. However, differential variation frequencies have also been obtained using different regeneration methods. Somatic embryo plants have generally been less variable, and that indicated the maintenance of genetic fidelity; however, somaclonal variation could be observed when somatic embryo cultures were kept for a longer time in *in-vitro* conditions (Henry *et al.*, 1997). Table 9, and other

Table 6. Effect of genotypes on somaclonal variation frequencies in ornamental plants

Species	Type of variation	Genotype	Frequencies (%)	Reference
Begonia	Phenotypic	cv. Schwabeland red	34.45	Westerhof *et al.*, 1984
		cv. Aphrodite pink	19.87	
Carnation, 1984	Early flowering	12 cvs	0.0	Gimelli *et al.*, 1984
		1 cv. (Rubino)	100	
		4 cvs	1.25–96.7	
Chrysanthemum	Floral morphology and colour	EC 7	35.0	Khalid *et al.*, 1989
		EC 8–EC 17	20.0	
		EC 23	15.0	
	Chromosome no. in regenerated plants	EC 23	20.0	
		EC 7–EC 8–EC 17	0.0	
Kalanchoe, 1988	Phenotypic variation	1 cv. (Montezuma)	1.8	Schwaiger and Horn, 1988
		1 cv. (Seraya)	28.3	
		4 cvs	8.8–15.7	
Pelargonium, 1976	Leaf phenotype	5 cvs	Significant differences	Skirvin and Janick, 1976a

Table 7. Effect of the source of initial explants on somaclonal variation frequencies

Species	Type of variation	Source of explant	Frequencies (%)	Authors
Carnation	Plant height (h2)	Meristems	20.29	Buiatti *et al.*, 1986
		Petals	142.83	
	Flowering date (h2)		24.89	
		Petals	34.91	
Chrysanthemum	Phenotypic variation	Leaf	0–10	Khalid *et al.*, 1989
		Petal		
	Flower colour	Shoot tip	7.0	
		capitula	22.0	
		stem segments	37.0	
	Chromosome number in regenerated plants	Capitula, shoot tip	0.0	
		Stem segments	36.0	
Marguerite	Dwarf habit	Shoot tip	0.0	Gimelli *et al.*, 1993
		Petal	15.8	
Pelargonium	Phenotypic variation	Nodal culture	0.0	Cassells and Carney, 1987
		Stem	16.0	
Ranunculus	Chromosome number and flower colour	Thalamus (embryogenesis)	0.0	Meynet and Duclos, 1990
		Anther (regeneration)	23.8	

reports on ornamental plants derived from *in-vitro* cultures, have consistently shown a steady increase in the occurrence of somaclonal variation (Preil, 1986). Thus, subcloning in *Haworthia* (Ogihara, 1990), an increase in the number of propagation cycles in *Begonia* (Westerhof *et al.*, 1984), and ageing of *Begonia* (Cassells and Morrish, 1987) and *Pelargonium* cultures (Bennici, 1974), all increased chromosomal and/or phenotypic variation among regenerated plants (Table 8). Finally, the culture conditions and the micro-environment during cell proliferation and plant regeneration play critical roles in the determination of mutation frequencies and also of generic variability spectra in some cases. Furthermore, the frequency of somaclonal variation also depends on the way of regeneration. As a rule, most variation is observed in plants originating from protoplasts, which is also termed protoclonal variation (see Kawata and Oono, 1997; Jain, 1997). Plants from unorganized callus vary more than from organized callus, whereas no, or hardly any, variation occurs when plants are regenerated directly without an intermediate callus phase. As a rule of thumb it should be noted that somaclonal variation occurs only in plants formed by adventitious regeneration, and not in plants formed by axillary branching (in which the original meristem remains intact) (De Klerk, personal communication).

Although no clear evidence exists on direct mutagenic effects of the culture media components in specifically aimed experiments such as those of Dolezel and Novak (1984), some data suggest that hormone balance and stress conditions may directly or indirectly influence genome dynamics during culture. Bogani *et al.* (1995, 1996) analysed genetic variation with RAPDs in tomato long-term cultures grown on media containing different auxin/cytokynin equilibria. In these experiments the rate of variability was affected by the media composition, but the optimal growth regulator balance for growth and regeneration yielded the lowest mutation frequency. Moreover, by using a mutual information coefficient designed to evaluate possible correlations among RAPD fragments, the authors found media-specific band patterns that were fixed in the long-term cultures. Similar behaviour was observed by the same authors when comparing patterns of cells selected for increased and decreased competence for active defence against *Fusarium oxysporum*. These cells had physiological phenotypes, which suggested the key differences in growth regulator balance between the two types of cell clones.

The significant effects of concentrations of the plant growth regulators on somaclonal variation were demonstrated in strawberry cultures by Nehra *et al.* (1992) and in ornamental plants (*Begonia* and *Saintpaulia* by Jain (1993c) who, however, found no qualitative differences in mutation spectra between treatments. Finally, a recent review (Buiatti and Bogani, 1997) indicated that RAPD analysis showed, in a number of cases, the appearance of fragments specific to treatments with osmotic stress factors.

Practical Uses of Somaclonal Variation in Ornamentals

So far, the reported data suggest that, particularly in long-term cell and tissue cultures, new genotypes adapted to the culture conditions may become fixed. This

supports the possibility of using *in-vitro* techniques for the selection of qualitatively and quantitatively improved traits, eventually leading to the release of improved cultivars. Therefore, *in-vitro* culture may be considered as a mutagenic treatment and its use can be compared with mutation breeding (Buiatti, 1989). Classical mutation breeding suffers from two serious drawbacks. Firstly, most mutations are recessive; therefore, it is difficult to improve characters where gene expression is necessary and they are generally dominant (for example resistance to pathogens) traits. Secondly, no easy selection techniques of the mutagenized materials are available. In most cases, isolation of favourable materials is often a matter of chance. The use of *in-vitro* cultures may partially allow one to overcome the first problem because somaclonal variants are often dominant than induced mutants with 'classic' mutations. Theoretically, *in-vitro* selection could be conducted on large cell population density, provided the right selection agents are used. Unfortunately, however, the last condition has not been generally met with the exception of the selection limited against resistance to abiotic and biotic stresses. Another problem with *in-vitro* selection protocols is that the selection requires a fairly long period in the culture, and this inevitably causes an increase in the frequency of unwanted mutations, and often a difficulty in regeneration of a large number of plants (Griesbach, 1988; Roest and Gilissen, 1989). Finally, selected desirable phenotypes may be of epigenetic origin and, therefore, may revert to normal after meiosis, or even following a few generations of vegetative propagation. As observed by several authors (e.g. Benetka and Kodytek, 1988) coupling classic mutagenesis with *in-vitro* culture (*in-vitro* mutagenesis), hoping to increase mutation frequencies and widen mutation spectra, has not changed the end results very much.

All this notwithstanding, some encouraging results have been obtained with the selection of genotypes resistant to stresses in many plants (Buiatti, 1989) and also in ornamentals (Table 9). In this field, hopes had been raised in the 1980s by sporadic good results obtained using fungal toxins or culture filtrates as selective agents against pathogens for resistance. However, Buiatti and Ingram (1991) emphasized that this technique covers only one of the aspects of defence from pathogens, and the selected phenotype may not be transmitted to the progeny. A few methods have been developed for indirect or negative selection for the competence for active defence based on improved pathogen recognition by the host, which may offer good opportunities in the future (Storti *et al.*, 1989). Some interesting results have already been obtained in the selection for resistance to abiotic stresses in ornamentals (Table 9), to low temperatures and high salinity, two characters of relevance for today's ornamental production. Moreover, few mutations will definitely survive all types of natural stresses, and their economic value is very much dependent on the plant species. In cereals, dwarf mutants are advantageous in monocultures but disadvantageous in nature; or mutants tolerant to disease and drought, or in other words the survival value of mutants is important in the field. In ornamental plants, survival value of a mutant is not as essential as in other crop species because ornamental plants are normally grown and maintained indoors. Any mistake by a tailor may become a new fashion in the textile fashion industry. Similarly, a weird-looking ornamental mutant may have more survival

Table 8. The enhancing effect of *in vitro* culture duration on mutation frequencies

Species	Type of variation	Culture duration	Frequencies (%)	Authors
Begonia	Phenotypic variation	One cycle	4.2	Westerhorf *et al.*, 1984
cv. Schwabeland		Two cycles	23.7	
Red		Three cycles	45.2	
cv. Lucille 1987	Polyploid plants	Callus subcultured	0.0	Cassell and Morrish, 1987
Closon		Callus aged	12	
Haworthia	Karyotypes in regenerated plants	Original callus culture	10.8 (abnormal karyotypes)	Ogihara, 1990
		Subclones	57.8 (abnormal karyotypes)	
	Leaf shape and esterase zymograms	Original callus culture	Significant differences	Ogihara, 1990
		Subclones		
Pelargonium (haploid)	Nuclear conditions in cells of *in-vitro* roots	Young callus (2 months)	2n = 71.42; 3n = 0; 4n = 0	Bennici, 1974
		Old callus (9 months)	2n = 13.81; 3n = 17. 4n = 44.82	

value and could become a useful ornamental cultivar. A similar mutant in other crops may not have high survival value. Therefore, mutation breeding is likely to be more important in ornamental crops.

Conclusions

A complete evaluation of the real impact of the use of somaclonal variation in ornamental breeding is severely hampered by the fact that *in-vitro*-based techniques have been routinely introduced into the breeding practice of a number of private enterprises, and do not represent a novelty. This, while offering the best proof of the application of *in-vitro* culture techniques, does not allow us to conclude any real cost–benefit ratio on the basis of advantages and disadvantages of somaclonal variation for breeding. However, published evidence of new genetic variability does indeed exist (Table 9) and some tentative conclusions on a few established facts can be drawn. First, it seems to be established that adventitious regeneration does increase the instability of plant genome. Second, the mutation spectra observed in regenerated plants do not entirely overlap with spontaneous or mutagen-induced genetic variability. Cytological and molecular analyses of *in-vitro* cell clones and regenerated plants do confirm this hypothesis, revealing increased frequencies of phenomena such as DNA amplification, permanent changes in methylation patterns, transposon activation, gene, genome and chromosome mutations. Third, the extent of recovered genetic variability is highly dependent on a series of factors such as the genotype (Table 6), the explant origin (Table 7), culture conditions, duration of the undifferentiated state, regeneration process, etc. This allows a certain amount of control modulation of mutation frequency. All these facts, therefore, suggest that somaclonal variation can be used as an additional source of selectable heritable variability, particularly in vegetatively propagated plants, either alone or in combination with more conventional methods, e.g. physical or chemical mutagenesis. Moreover, the existing evidence offers a limited set of tools for *in-vitro* selection for a few desired traits, and for the modulation of overall mutation frequencies. This is particularly relevant for avoiding unwanted variability in conventional breeding and when recombinant DNA techniques are being used.

Serious drawbacks to implementation of utilization of somaclonal variation as a breeding tool still exist, and should be emphasized here. Firstly, some species and genotypes show little or no variability and the regeneration techniques are often limited to one or a few genotypes. This is one of the factors of limited use of direct *in-vitro* selection techniques. Another reason is the lack of useful markers, generally due to poor information on the physiological basis of economically important traits. Finally, in most cases, unwanted genetic variability is recovered together with improvement of desired traits, a factor which tends to increase the cost and duration of ornamental breeding.

It may be worth recalling at this point that progress in the biological sciences has provided breeders with a number of new tools, all of which have been

Table 9. In-vitro selection techniques in onamental plants

Species	Selected tissues	Selection agent	Results	Authors
Ficus lyrata, 1991	Not reported	None	Variegated	Garcia and Moreno
African violet	Leaf callus	Low temperature (15°C)	Low temperature tolerance (LTT)	Grunewaldt, 1988
Chrysanthemum	Cell suspensions	Low temperature (8°C)	LTT	Preil *et al.*, 1983
		Sub-optimal temperature	LTT	Broertjes and Lock, 1985
	Cell suspensions	Low temperature (6°C)	Early flowering	Pluitema *et al.*, 1991
	Internode callus	Low temperature 8°C	LTT	Benetka and Kudytek, 1988
Coleus	Callus	NaCl	Increased resistance in seed progeny	Ibrahim *et al.*, 1992
	Callus	NaCl	Cell line resistant to salt tolerance	Collins *et al.*, 1990
Fuchsia	Leaf callus	Low temperature (13°C)	LTT	Bouharmont and Dabin, 1986
Poinsettia	Cell suspensions	Temperature stress (12°C)	LTT	Preil *et al.*, 1983 Walther and Preil, 1981

LTT, low temperature treatment.

envisaged, at the time of their discovery, as totally revolutionary and could become an alternative to 'conventional' practices. This happened with the discovery of heterosis (hybrid vigour), induced polyploidy, methods for the creation of new species through interspecific hybridization, mutagenesis and, more recently, recombinant DNA techniques. None of these tools has proven to change agriculture drastically when applied in a vacuum. Eventually, after the excitement and enthusiasm of the discovery and the consequent disillusionment, they have all been included in the breeding practices with fruitful results.

It is fair to conclude that somaclonal variation, if exploited alone, is far from being a magic source of solution to the problems of ornamental plant breeding. Nevertheless, it has proven to be a useful additional tool with limited genetic systems and/or narrow genetic bases, where it can provide a rapid source of variability for crop improvement (Karp, 1995). Somaclonal variation will continue to be used to obtain new and improved genotypes of agronomic and economic value.

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6. Somaclonal Genetics of Forest Trees

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Introduction

In addition to conventional mutation breeding approaches to increase genetic variation for crop improvement, plant tissue cultures also offer opportunities for recovering regenerants with genetic variability. Nearly all types of genetic change detected in plants following irradiation or chemical treatments have also been observed in plant tissue cultures. However, in plant tissue cultures the spectrum of genetic variation may be additionally enhanced by treatment of cultured cells with chemical and physical mutagens (Ahloowalia, 1995; Skirvin *et al.*, 1994). Variation has been observed for phenotypic traits, chromosome number and structure, and biochemical traits in plant tissues/regenerants derived from cells, protoplasts, anthers, or other tissues. However, it must be stated at the outset that somaclonal variation has not considerably enhanced the spectrum of useful mutations that have been produced by mutation breeding. Not all the variation produced in tissue cultures is due to mutations; some of it results from paragenetic (epigenetic) changes. Although, variation in plant tissue cultures has been observed for a long time, only in recent years was it categorized and its importance recognized. Plants regenerated from callus have been termed 'calliclones' (Skirvin, 1978), and those regenerated from protoplasts as 'protoclones' (Shepard, 1981). Larkin and Scowcroft (1981) proposed a more general term, 'somaclones', for plants derived from any type of somatic cell culture, and genetic variability originating from such cultures as 'somaclonal variation'. In order to distinguish between variants regenerated from somatic or gametic cell cultures, Evans *et al.* (1984) have proposed an additional term, 'gametoclonal variation', which may arise in haploid cell cultures derived from gametic cells. Variation occurring in the primary regenerants derived by *in-vitro* genetic transformation has been termed 'somatoclonal variation' (Ahuja, 1988a). The origin and causes of variation in plant cell cultures are not well understood; but it is likely that a number of different factors, genetic and non-genetic, are involved in this process.

Ever since somaclonal variation was introduced as a novel source of variability in cell cultures for plant improvement, by Larkin and Scowcroft in 1981, it has attracted considerable attention in non-woody species (see Bajaj, 1990, 1996; Cullis and Greissen, 1987; Evans *et al.*, 1984; Evans and Sharp, 1986; Gould, 1986; Krikorian *et al.*, 1989; Phillips *et al.*, 1994; Skirvin *et al.*, 1994), as well as in woody plants (see Ahuja, 1987a; Chen and Ahuja, 1993; Fry *et al.*, 1998; Michler, 1993; Ostry and Michler, 1993; Skirvin *et al.*, 1993). In this chapter I shall briefly examine the sources and spectrum of somaclonal variation, but focus mainly on its genetic basis and its application to forest trees.

S.M. Jain, D.S. Brar, B.S. Ahloowalia (eds.), Somaclonal Variation and Induced Mutations in Crop Improvement, pp. 105–121.

Nature of Variation in Somatic Tissues

Variation originating in the somatic tissues grown *in vitro* may result either from altered phenotype, or genotype, or both (Michler, 1993). Phenotypic variants may either revert to the original normal type after removal from the *in-vitro* environment, or the variant may continue to exhibit the new phenotype in the primary regenerants and their clonal propagules. The origin of the new variant may be traced to a gene mutation(s) or an epigenetic change(s). Mutations are usually rare and spontaneous events, which occur at rates lower than 10^{-5} per cell generation in the cells. Mutations are caused by changes in gene structure, and are generally irreversible. The epigenetic events, on the other hand, involve developmental changes which occur at a relatively high frequency of 10^{-3} per cell generation (Meins, 1989), and are caused by altered expression of genes, without changes in gene structure, and are potentially reversible. The epigenetic events are directed changes; that is, they occur regularly in response to inducers (Meins, 1983).

Although, variation in tissue culture may result from mutations (gene mutation, chromosome rearrangements or deletions) or epigenetic changes, it is possible to discriminate between the two types of events on the basis of progeny tests. If the new trait appears in a somatic cell, and is transmitted to its daughter cells and to further generations of somatic cells in an organism in a stable state, then the trait may be considered genetic. However, only that trait is considered hereditary whose predisposition is transmitted from the parents to the offspring or progeny via sexual hybridization. In the present context the genetic changes involving mutations which are passed on, via hybridization, from one generation to the next, are hereditary, whereas the epigenetic changes affecting gene expression are normally not transmitted through meiosis to the progeny, and therefore are not heritable. In annual crop plants, the hereditary or epigenetic basis of the somaclonal variation can be easily determined by progeny tests. However, in long-lived woody plants, it is sometimes difficult to determine whether the genetic change is due to a mutation or an epigenetic event (if it persists in the somaclones) in the absence of a genetic cross. Since some of the epigenetic changes are transmitted in the somatic cell generations in the regenerant, the altered trait may be maintained in woody plants by vegetative propagation. Therefore, epigenetic somaclonal variation for an economically important trait may be exploited in woody plants, as long as the trait persists and is stably transmitted across the somatic cell generations by clonal propagation.

Designation of Somaclonal Progeny

In addition to these terms describing the type of variation arising in *in-vitro* cultures, it is also necessary to designate the progenies derived from regenerated plants cells. In order to follow the mode of inheritance of mutant genes in the selfed progenies of the somaclones, Chaleff (1981) propopsed the symbol R for the regenerant, R_1 for the progeny of the primary regenerant, and R_2 and R_3 for the successive selfed generations. However, this nomenclature system does not

correspond to the accepted genetic usage in which the segregation is first observed in the F_2 (hybridization) or M_2 (mutagenesis) generations. According to Chaleff's numbering series, a mutation present in the heterozygous state in the primary regenerant would first segregate in the R_1 generation. Recognizing this discrepency, Yurkova *et al.* (1982), who retained the R symbol, renumbered the series as R_1 for the regenerant (carrying the mutation in the heterozygous state), and R_2 and R_3 for the successive selfed generations. However, in cross-pollinated crops, including many forest tree species, the R_1 must be backcrossed to a recurrent parent to determine the nature of the mutant.

To minimize confusion between numbering systems in the R generations, Larkin *et al.* (1984) proposed a change from the R (regenerant) to SC (somaclone), and suggested SC_1 for the primary regenerant, and SC_2, SC_3 for the subsequent selfed generations. Although the letters R and SC, or just S (Fry *et al.*, 1998), have been used by different investigators for primary *in-vitro* regenerants, there is a need to standardize the nomenclature in this field. There are at least two possible ways to handle this situation. A simplified approach would be to designate somaclones derived from any type of tissue as SC or just S. On the other hand, it might be useful to approach the problem based on the tissue of origin. In that event, I would suggest that we retain the R designation for the regenerants from the somatic cells following the microshoot/root plantlet pathway, and propose the term SR for the regenerants derived from somatic cells via somatic embryogenesis, and PR (protoplast regenerants, or protoclones (Serres *et al.*, 1991; Shepard, 1981) as the primary regenerants from the protoplasts. In order to be consistent with the genetic usage in the zygotic progenies (F_1 and F_2), the series R_1 would represent the primary regenerant, SR_1 the primary somatic embryo-derived somatic plant, R_2 and SR_2 as their subsequent generations respectively (Ahuja, 1991a), PR_1 as the primary protoplast regenerants, and PR_2 and PR_3 as their later generations.

The question arises as to how to designate the propagules derived by successive generations of vegetative propagation from the primary regenerants. Since, according to the accepted genetic nomenclature system, the filial generations are designated by subscript numbering series (F_1, F_2, etc.), I propose that we designate the primary regenerant by the symbol R-1, and its clonally propagated subsequent vegetative generations as R-2, R-3 and so on. It is useful to follow the somatic cell genetics of those crops that are routinely propagated *in vitro* by vegetative means for a number of clonal generations. This is particularly important in long-lived woody plants in which one sexual generations may take one to several decades, depending on the species, but where several vegetative generations can be produced by tissue culture technology in less than a decade.

Spectrum of Somaclonal Variation in Plants

For obtaining true-to-type propagules *in vitro* from selected genotypes for clonal forestry programmes or germplasm preservation, somaclonal variation is undesirable. On the other hand, somaclonal variation offers prospects for recovery of

mutants in tissue cultures, some of which may be useful. Most of the variation observed in earlier studies indicated that chromosomal changes and ploidy alterations were more frequent in tissue culture regenerants in herbaceous crops (Ahloowalia and Sherington, 1985; Bayliss, 1980; D'Amato, 1978; Evans and Sharp, 1986; Gould, 1986; Karp and Bright, 1985; Skirvin, 1978; Sree Ramulu *et al.*, 1983). However, later studies have shown that heritable somaclonal variations involving single gene mutations also occur in the regenerants, for example tomato (Evans and Sharp, 1983), wheat (Larkin *et al.*, 1984), and maize (Brettell *et al.*, 1986). Variation has been observed in several traits: seed colour in mustard (George and Rao, 1983), fruit and leaf colour in tomato (Evans and Sharp, 1983), disease resistance in potato (Shepard *et al.*, 1980), and sugarcane (Larkin and Scowcroft, 1983), flower colour in alfalfa (Groose and Bingham, 1984), yield and agronomic traits, including height, tillering, and seed weight in wheat and rice (Ahloowalia and Sherington, 1985; Sun *et al.*, 1983), and organelle DNA in potato (Kemble and Shepard, 1984). Thus it is clear that plant cells/tissues and culture milieu interactions can lead to a fairly large spectrum of genetic variation in plants. Although, somaclonal variation has been detected in a large number of plant species, only in a few cases have critical genetic analyses been carried out to determine the heritable basis of the variation (Ahuja, 1987a, 1996; Evans, 1989; Evans and Sharp, 1986).

Somaclonal Variation and Selection in Trees

During the past two decades, considerable progress has been made towards *in-vitro* regeneration from woody plant callus, cell, organ, and protoplast cultures (Ahuja, 1982, 1984, 1988b, 1991b, 1993b; Ahuja *et al.*, 1996; Bonga and von Aderkas, 1992; Bonga and Durzan, 1987; Huang *et al.*, 1993; Jain *et al.*, 1995). Somaclonal variation has been observed in the regenerants of a number of woody plant species (Ahuja, 1987a; Fry *et al.*, 1998; Hammerschlag, 1992; Michler, 1993; Skirvin *et al.*, 1993, 1994). Perhaps Lester and Berbee (1977) were the first to systematically record variation in the *in-vitro* regenerants in trees. They observed a wide range of variation in height, number of branches, leaf traits and chromosome numbers in the callus-derived regenerants from black poplar (*P. nigra*) and Euramerican poplar (*P.* × *euramericana*). Subsequently, somaclonal variation for different traits has been observed in other species of *Populus*, as well as a number of other woody plant genera. Somaclonal variation was reported for leaf morphology and thickness within poplar clones from different sections of the genus *Populus* (Antonetti and Pinon, 1993), and growth, leaf phenotype, and leaf gas exchange characteristics of poplar hybrid (*Populus tricocharpa* × *P. balsamifera*) TT32 lines regenerated after prolonged callus cultures (Saieed *et al.*, 1994a,b). The spectrum of variation in the primary regenerants (R-1) in the poplar TT32 lines was stably expressed in the R-2 vegetative propagules (Fry *et al.*, 1998). Variegated leaves have been observed in the *in-vitro* regenerants of *Liquidamber styraciflua* (Brand and Lineberger, 1988), and *Paulownia*

tomentosa (Marcotrigiano and Jagannathan, 1988). Variegated plants and differences in root morphology have been observed in the clones regenerated from bud cultures of *Populus tremula* (Ahuja, 1983). In addition, changes in ploidy levels have been been reported in poplar callus cultures (Son *et al.*, 1993) and regenerants, which were fully or partially tetraploid, and exhibited leaf traits characteristic of polyploids (Antonetti and Pinon, 1993; Jehan *et al.*, 1994; Saieed *et al.*, 1994b).

Somaclonal variation has been observed for salt, herbicide and disease tolerance in woody plants. Callus cultures and regenerants tolerant to salt (sodium chloride) have been recovered in grape (Skene and Barlass, 1983), *Citrus* (Ben-Hayyim and Goffer, 1989; Spiegel-Roy and Ben-Hayyim, 1985), and colt cherry (*Prunus avium* × *P. pseudocerasus*) (Ochatt and Power, 1989). Regenerants from cell suspension cultures and leaf explants of a hybrid poplar were screened for tolerance to two herbicides, glyphosate and sulphometuron methyl, and several herbicide-tolerant somaclonal selections were made for greenhouse and field tests (Michler and Bauer, 1988; Michler and Haissig, 1988; Michler *et al.*, 1989). Putative resistance to diseases, using somaclonal selections, were observed for *Septoria* leaf spot in hybrid poplars (Ostry and Skilling, 1988), *Melampsora* leaf rust in cottonwood (Prakash and Thielges, 1989), *Hypoxylon* canker in *P. tremula* (Valentine *et al.*, 1988), and *Xanthomonas* in peach (Hammerschlag, 1990).

Although, gymnosperm cell cultures have been considered relatively more stable than angiosperms (Diner and Karnosky, 1987), somaclonal variation has also been observed in some of its genera. Micropropagated plants from loblolly pine (*Pinus taeda*) showed a higher mortality, less shoot growth, and more curved stems, as compared to seedlings (Leach, 1979), possibly due to poor root system (McKeand and Allen, 1984). Karyological variation has been observed in the callus cultures of *Abies alba* (Gajdosova and Vookova, 1991). Somaclonal variation in somatic embryo-derived plantlets has been observed in white spruce (*Picea glauca*) (Isabel *et al.*, 1995). Somatic embryogenesis-related somaclonal variation was also observed in Norway spruce (*Picea abies*) by Fourré *et al.* (1996). Cytological analysis revealed trisomy, double trisomy, tetraploidy, or mixaploidy in 5-year-old somatic embyrogenic masses. In addition, trisomy, and chimaeras with trisomic buds, but diploid roots, were also identified in the somatic plants of Norway spruce. However, molecular approaches using a large number of DNA primers to assess RAPDs (randomly amplified polymorphic DNAs) profiles revealed no intraclonal variation in a large sample of somatic embryogenic masses, somatic seedlings, and somatic plants of Norway spruce; however, it was possible to distinguish between four somatic embryo-derived clones (Fourré *et al.*, 1996). By employing RAPDs, intraclonal variation was also not observed in somatic embryos or somatic plants of Norway spruce (Heinz and Schmidt, 1994), and in *Picea mariana* (Isabel *et al.*, 1993). However, mitochondrial DNA variation, based on restriction fragment length polymorphisms (RFLPs), was detected in somatic embryogenesis of *Larix* species (De Verno *et al.*, 1994). Therefore, it would appear that RAPDs alone may not adequately detect intraclonal genetic variation but, combined with RFLPs and other co-dominant

molecular markers, such as SSR (short sequence repeats), it might be possible to increase the resolving power to detect mutations.

Gametoclonal Variation in Trees

Haploids have been produced in angiosperms mostly by culture of anthers (androgenesis), and in gymnosperms from female gametophytes. In both systems, the haploid cells may follow one of two pathways for plant regeneration: organogenesis or embryogenesis. The induction and establishment of haploidy in forest trees has been reviewed elsewhere (Baldursson and Ahuja, 1996a; Bonga *et al.*, 1988), and will not be further detailed here. Here, I shall discuss the potential of haploidy in relation to gametoclonal variation in woody plants. Variation has been observed for chromosome numbers, morphology, and vigour (Baldursson and Ahuja, 1996b). More often, a mixture of haploid and diploid regenerants with aneuploids and polyploids in different proportions have been observed in pollen cultures of *Populus* (Chen, 1986, 1987; Ho and Raj, 1985; Stoehr and Zsuffa, 1990a; Uddin *et al.*, 1988; Wu and Nagarajan, 1990), *Hevea brasiliensis* (Chen, 1986, 1987, 1990), and *Litchi chinensis* (Lianfang, 1990). Calli obtained from megagametophytes have usually been diploid, but in some cases higher ploidy has been observed in *Picea abies* (Huhtinen *et al.*, 1981; Simola and Honkanen, 1983), and *Picea sitchensis* (Baldursson *et al.*, 1993a).

Cytological studies have been conducted on trees derived from anther cultures. In *Hevea brasiliensis*, which is an allotetraploid species (2n = 36), 56% of the cells in the young leaves of plants less than 50 cm in height were dihaploid (2× = 18), and about 15% of the cells with 9 (×) or 27 (3×) chromosomes, and occasionally aneuploid and tetraploid (4× = 36) were also observed (Chen, 1990). In bigger plants (more than 150 cm), 18% of the cells were tetraploid, and this tendency increased as these plants grew older. Chromosome counts on the somatic cells of pollen-derived plants in *Populus* × *simonigra* and *P. berolinensis* similarly revealed a tendency towards mixoploidy at the time of transplanting, with diploid cells gradually increasing in frequency over time.

Variation in morphology has been observed in the form of anomalous flowers (mostly males) in the anther-derived plants of *Populus maximowiczii* (Stoehr *et al.*, 1988; Stoehr and Zsuffa, 1990b), *P. deltoides* (Uddin *et al.*, 1988), albino shoots in *Aesculus hippocastanum* (Radojevic and Trajkovic, 1983), and variation in height, leaf morphology, and vigour in *Hevea brasiliensis* (Chen, 1986, 1987, 1990; Chen and Ahuja, 1993), and *P. trichocarpa* (Baldursson *et al.*, 1993b). The question is whether the variation in anther-derived plants is culture-induced, or whether it might be due to direct expression of different genotypes resulting from recombinational and segregation events at meiosis. In the plants derived from haploid cells, recessive genes are expressed that will not be expressed in the heterozygous diploid state. Since the anther plants are not derived from single pollen grains, but from a mixture of pollen grains having different genetic constitution, it is possible that only part of the variation might be gametoclonal (Baldursson and

Ahuja, 1996b). On the other hand, plants derived from single megagametophytes or pollen may be more informative on the origin of gametoclonal variation.

Since trees are long-lived, there may be tendency for the gamete-derived plants to go through a mixoploid phase and then adjust to the diploid state over time. This adjustment might initiate after the gamete-derived plants are transplanted under field conditions and continue as the trees grow older. The majority of pollen-derived plants of *Populus trichocarpa*, obtained by direct microspore embryogenesis, appeared to be stably diploid (Baldursson *et al.*, 1993b). Trees derived from the megagametophytes of *Sequoia sempervirens* when transplanted to the field were all diploids. A few haploid plants of *Larix* spp. grew for a while in soil, but then they died (Von Aderkas *et al.*, 1990). However, one plant of gametophytic origin in *Larix* was mixoploid with a predominance of diploid cells, and exhibited a plagiotropic growth in soil (von Aderkas and Bonga, 1993).

Somatoclonal Variation

The variation occurring in transgenic plants that seems to be associated with *in-vitro* transformation and gene transfer events has been termed 'somatoclonal variation' (Ahuja, 1988a). We might ask the question: Does tissue culture also contribute to genetic instability of transgenic plants? If so, then it is possible that transgenic plants may inherit genetic instability from two sources: one source of variation may be from the tissue culture itself, and the second source following transfer of a foreign gene. Somatoclonal variation may be heritable or epigenetic, and it may be due to one or more events occurring singly or in concert. These include: copy number of recombinant gene inserted in the host genome, site of integration of transgenes, position effect, loss of a foreign gene, and loss of transgene expression (Ahuja, 1988c,d, 1998). Recent reports on herbaceous crops indicate that transgene expression is less stable in transgenic plants. Gene silencing, that is, partial or complete inactivation of recombinant genes as well as endogenous genes, has been observed in a number of herbaceous transgenic plants. Inactivation of transgenes may occur either at the transcriptional or post-transcriptional level (see Finnegan and McElroy, 1994; Flavell, 1994; Jorgensen, 1995; Matzke and Matzke, 1995; Meyer, 1995). Degrees of transgene expression are also variable between transformants, and this variability may be conditioned by the copy number and/or site of integration of the transgene (Hobbs *et al.*, 1990, 1993; Pröls and Meyer, 1992).

Forest trees are long-lived and have vegetative phases that might extend from one to several decades in different species. Since forest trees are anchored on one location for their entire life, they are constantly exposed to changing environments over long periods of time, which may influence their physiology, growth, and complex morphogenetic processes. Under such conditions, those genes that confer low fitness in trees, for example some recombinant genes, may either be silenced, or turned on at unexpected times, or eliminated. Therefore, gene transfer in trees needs much further investigation for a better understanding of genetic

stability of transgenes on a short-term and long-term basis. A number of questions on the genetic instability of transgenes present themselves that have relevance to somatoclonal variation in long-lived forest trees (Ahuja, 1988a,c,d). Are the recombinant genes stably integrated and expressed immediately in a specific tissue or in all tissues in woody plants? Do site-specific promoters exhibit differences in transgene expression? Are the transgenes expressed immediately, but afterwards remain inactive/silent for a long time, and are then re-expressed? Do transgenes undergo rearrangements, or are they lost during the long vegetative phase of trees? Do transgenes cause genetic changes in the host genome by position effect at different sites in the genome? These questions need to be addressed for more insight into somatoclonal variation in woody plants.

Recombinant genes have been transferred in a number of woody plant species (Charest and Michel, 1991). However, only in a limited number of genera, including poplar (*Populus*), walnut (*Juglans*), sweetgum (*Liquidambar*), neem (*Azadirachta*), larch (*Larix*), spruce (*Picea*), casuarina (*Casuarina*), apple (*Malus*), citrus (*Citrus*), and plum (*Prunus*) have transgenic plants been produced (Dandekar, 1992; Jouanin *et al.*, 1993). Of these genera, only a couple, in particular *Populus*, have been tested under greenhouse and field conditions (Ahuja, 1998; Ahuja *et al.*, 1996; Pardos *et al.*, 1994). Because of the relative ease of *in-vitro* regeneration and transformation by the *Agrobacterium* vector system, transgenic plants have been produced for several species and species hybrids of the genus *Populus* (Ahuja and Fladung, 1996; Donahue *et al.*, 1994; Fillatti *et al.*, 1987; Fladung *et al.*, 1997; Jouanin *et al.*, 1993; Klopfenstein *et al.*, 1993; Tsai *et al.*, 1994).

A number of recombinant genes, including those for herbicide tolerance (Donahue *et al.*, 1994; Fillatti *et al.*, 1987), lignin modification (Boerjan *et al.*, 1996; Whetten and Sederoff, 1991), sterility (Strauss *et al.*, 1995), histochemical effects (Ellis *et al.*, 1996), and phenotypic effects (Ahuja and Fladung, 1996; Fladung *et al.*, 1997) have been transferred to woody plants.

A first report on genetic transformation by *Agrobacterium tumefaciens* in woody plants involved transfer of the *aroA* gene from *Salmonella* for herbicide resistance in a hybrid poplar (*Populus alba* × *P. grandidentata*) clone NC 5339 (Fillatti *et al.*, 1987). There was not only variation in the type of shoots produced *in vitro* following genetic transformation (Fillatti *et al.*, 1987), but also expression of the *aroA* gene was less strong than expected, when transgenic poplars were sprayed with the herbicide glyphosate (Riemenschneider and Haissig, 1991; Riemenschneider *et al.*, 1988). Although in a recent study, Donahue *et al.* (1994) have demonstrated enhanced expression of the *aroA* gene under the control of 35S promoter, as opposed to the mas promoter employed by Fillatti *et al.* (1987) in hybrid poplar, there was inadequate discussion on genetic stability of the transgenic poplars.

The expression of another recombinant gene *uidA* (GUS) under the control of different promoters has been investigated in hybrid poplar and spruce (Ellis *et al.*, 1996; Nilsson *et al.*, 1996). In the *rolC–uidA* transgenic hybrid aspen, the GUS activity was tissue-specific and mainly localized in the vascular tissue, and showed seasonal changes, shifting during winter dormancy from vascular to pith

and cortex, and finally disappeared in the stem tissues. By contrast, GUS expression in the stem of 35S–*uidA* transgenic hybrid aspen was strong in all tissues except the vascular cambium and xylem, and did not vary in intensity during growth and dormancy (Nilsson *et al.*, 1996).

Variation in GUS expression in young leaves, as compared to mature leaves, of 35S-*uidA* transgenic hybrid poplar clone NC5339 was also detected during the growing season. On the other hand, GUS activity was relatively higher in the older than younger leaves in another poplar hybrid (*P. nigra* × *P. maximowiczii*) clone NM6. In white spruce (*Picea glauca*), GUS activity was detected only during needle elongation, but in the stems it persisted throughout the growing season. Based on these studies on poplar and spruce, Ellis *et al.* (1996) concluded that GUS expression was least variable during the growth of *in-vitro* regenerated transformants, but most variable in field-grown transgenic plants. In all transgenics in the field, GUS activity was the highest in early spring just after bud break, but then the activity rapidly decreased. The suppression of GUS expression was observed in a small number of transgenic lines in both poplar and spruce. In addition, a large variation in transgene expression was observed between individuals from the same population.

In a recent investigation, we have employed *rolC* gene from *Agrobacterium rhizogenes* under the expressive control of 35S (from cauliflower mosaic virus) and rbcS (from potato) promoters (35S–*rolC* and rbcS–*rolC*) for genetic transformations of European aspen (*Populus tremula*) and hybrid aspen (*P. tremula* × *P. tremuloides*) clones (Ahuja and Fladung, 1996; Fladung *et al.*, 1997). The *rolC* gene is expressed at the phenotypic level by reduction in leaf size (mainly in 35S–*rolC*) and chlorophyll content (pale green colour), and stimulation of rooting, as compared with the untransformed aspens. Therefore, the *rolC* gene offers prospects for monitoring its altered expression during growth and development in transgenic plants, and consequently questions regarding genetic stability of transgenes in woody plants at the phenotypic and molecular level. Transgenic plants carrying other recombinant genes are also suitable for monitoring genetic stability, but because of their lack of effect on the phenotype, their expression must be analysed by exposure to specific agents, for example a herbicide or a pest, at regular intervals, as well as by biochemical/molecular markers. By employing the *rolC–Populus* system, we have detected a number of phenotypic abnormalities, including chimaeric leaves and plants, revertants to the normal state in the R-1 and R-2 generations. In addition, a couple of transgenic plants which were positive for the *rolC* at the molecular level (PCR amplification), were negative for the *rolC* phenotype (Fladung and Ahuja, 1997 and unpublished). Southern blot analysis, employing a non-radioactive hybridization method (Fladung and Ahuja, 1995), revealed that most of the transgenic aspens carried a single copy of the transgene. However, some transgenic aspens carried two or more copies of the *rolC* gene (Fladung *et al.*, 1997), and these plants showed lower survival rates. We are continuing the molecular analysis of the transgenic aspens showing phenotypic abnormalities, and are examining the mechanisms of transgene inactivation in aspens.

These studies with transgenic woody plants, obtained by using genetic transformation by a number of different recombinant genes having different reporter genes and promoters, indicate that transgene expression depends on the type of promoter, the genotype of the plant, the copy number of the transgene, and environmental conditions under which transgenic plants are grown. The degree of somatoclonal variation would be conditioned by the genetic constellation of the transgene in the tree genome and the environmental factors. In the absence of progeny tests, it is difficult to ascertain whether somatoclonal variation is heritable or epigenetic in long-lived, transgenic woody perennials.

Origin and Mechanisms of Somaclonal Variation

Ever since the introduction of the term 'somaclonal variation' in 1981, by Larkin and Scowcroft, it has been the subject of many reviews. However, the mechanisms underlying somaclonal variation are still not fully understood. Based on the available data from tissue culture of a large number of plants, including woody perennials, it appears that a number of different processes, singly or in combination, may be involved in the origin of somaclonal variation. The spectrum of somaclonal variation, including gene mutations, chromosome aberrations, parallels the type of mutations induced by physical or chemical agents in plants. One might ask what is unique about tissue culture and its ability to induce a wide range of genetic variation, in the absence of known mutagens in the medium? One could argue that the variation is already present in the plant tissues before culture, and the *in-vitro* milieu simply sorts out genetic or epigenetic changes. Plant tissues are composed of a heterogeneous array of cells of various ages, different physiological states and degree of differentiation, and may occasionally contain cells with different ploidy levels. The molecular states in the genomes of such heterogeneous cell types may be at flux in terms of gene expression. By placing cells in tissue culture, the genome at different molecular states is suddenly under stress to cope with *in-vitro* condition. Cellular survival depends on the ability to adapt to the new situation. In doing so, the cellular genome becomes error-prone. Alternatively, when cells are removed from their normal conditions and placed in a tissue culture environment, the genome undergoes restructuring as a response to the new stress conditions (McClintock, 1984). The relevant mechanism for the origin of genetic instability in tissue culture may involve programmed loss of cellular control (Phillips *et al.*, 1994). The end effect seems to be an array of genetic/epigenetic changes.

Plants regenerated from callus cultures of most species seem to be more prone to induction of genetic variation. It turns out that somaclonal variation occurs at a relatively higher frequency in cultured cells as compared to spontaneous mutations in uncultured cells. The most commonly observed changes in callus cultures and their regenerants are in chromosome number structure (rearrangements) (D'Amato, 1978; Bayliss, 1980; Bonga, 1981), gene mutations, and epigenetic changes involving DNA methylation (Brown, 1989; Kaeppler and Phillips, 1993;

Smulders *et al.*, 1995). In addition, repeat-induced mutation (RIP), that is, point mutations occurring in the newly methylated sequences as the result of deamination of the methylated cytosine, may also contribute to genetic instability of tissue-cultured induced variation (Phillips *et al.*, 1994). Chromosome aberrations could be induced by altered levels of DNA methylation, and may include translocations, inversions, deletions, and duplications. Heterochromatization of chromatin may also be associated with increased methylation. Although, epigenetic changes are not normally heritable, certain methylation patterns may not only persist in the clonal generations from the R-1 regenerant, but may also be partly transmitted to the selfed R_2 progeny, as recently reported in tomato (Smulders *et al.*, 1995).

Several factors may influence the induction of somaclonal variation in plants. These include the genotype; the age of the donor plant; the explant source (stem segments vs. meristems, or leaf discs, or root segments); the ingredients of the medium, particularly hormones; the length of time tissues are kept in culture; and the cultural regimes (Ahuja, 1987a; Fry *et al.*, 1998; Karp and Bright, 1985; Skirvin *et al.*, 1994). Exposure of cells to relatively higher levels of exogenous hormones, such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), cytokinins, and other chemicals in the culture medium, which are several-fold higher than the physiological endogenous concentrations of these substances required for growth and differentiation, may play a major role in the induction of somaclonal variation. In particular 2,4-D, and other callus-inducing auxins, may be involved in increased methylation, and may induce RIP in the regenerants (Kaeppler and Phillips, 1993; Phillips *et al.*, 1994).

Outlook

Although, somaclonal variation offers prospects of recovering useful variants from cultured cells, most variation arising from *in-vitro* cultures is undesirable. Since tissue culture is a prerequisite for most currently employed gene transfer technologies, it is conceivable that pre-existing somaclonal variation may contribute an additional genetic load to somatoclonal variation. Therefore, it is important to standardize protocols and employ organ cultures (for example, meristems, leaf discs), which undergo direct differentiation, with minimal or no callus formation (Ahuja, 1986, 1987b, 1993a), and may consequently minimize the risk of somatoclonal variation in clonal regenerants. In this respect, somaclonal variation and somatoclonal variation are both associated with the occurrence of genetic variation. However, somaclonal variation is looked upon as a new source of desirable genetic variation; somatoclonal variation, on the other hand, is the source of genetic instability, which would be undesirable in transgenic plants, as well as their somatic regenerants or progeny. Because of long generative cycles with extended vegetative phases, woody plants offer prospects for monitoring the stability of the new somaclonal selections, as well as instability of somatoclonal regenerants on a short-term as well as long-term basis.

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7. Gametoclonal Variation in Crop Plants

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Introduction

Gametoclonal variation has been defined as the variation among plants regenerated from gametic cells in culture (Evans *et al.*, 1984; Morrison and Evans, 1987). Therefore, it can be due to changes that result from the culture process itself or to genetic recombination that preceded the formation of the gametes in an inbred, F_1, F_2, or otherwise heterozygous individual. A separation of these two origins of gametoclonal variation is possible only when gametes have been derived from homozygous lines, and are therefore expected to have little or no variation due to genetic recombination or by comparison of genetic variances of differently derived populations, e.g., doubled haploids (DH) vs. single seed descent (SSD). Attempts can be made to direct gametoclonal variation by imposition of selection agents during the process of haploid derivation. Also, for self-pollinated species, 'hybrid sorting' can be imposed by deriving haploids from the F_1 hybrid between two parents that differ by various agronomic attributes. This would provide inbred lines after one generation that should resemble SSD derivatives after many generations. Gametoclonal variants may also express undesirable mutations or ploidy changes.

An argument has been made that haploidization processes are valuable to the breeder only if the resulting plants represent the normal gametic array expected in sexually derived progeny. However, this may be true only in studies of genetic control of traits or segregation analyses. Agronomically superior gametoclones may be selected even from populations exhibiting skewed segregation. Some researchers have concluded that the ill-effects of gametoclonal variation can easily be ignored by selection of only the most vigorous or agronomically superior derivatives of haploidization procedures. Since the review article by Morrison and Evans (1987), several additional reports have been published on the occurrence of gametoclonal variation, whether to isolate superior plants or simply to document the phenomenon. This chapter focuses on publications subsequent to this review. The genetic bases of gametoclonal variation, recently reviewed by Logue (1996), include chromosomal numerical changes (polyploidy, aneuploidy); chromosome structural changes (duplications, deletions, inversions, translocations and interchanges); molecular alteration of the nuclear genome; and cytoplasmic changes (alteration of chloroplast or mitochondrial DNA). The reader is referred to Logue (1996) for a recent documentation of these events during androgenesis.

S.M. Jain, D.S. Brar, B.S. Ahloowalia (eds.), Somaclonal Variation and Induced Mutations in Crop Improvement, pp. 123–133.

Segregation of Molecular Markers

Abnormal segregation of molecular markers in anther-derived populations has been not only a concern that such populations may not be used to derive accurate genetic maps but also a possible key to determining genetic control of androgenesis. One result has been that genetically identical individuals have been found among anther-derived plants of highly heterozygous maize (Murigneux *et al.*, 1993a) and potato genotypes (Rivard *et al.*, 1989; Veilleux *et al.*, 1995). This has demonstrated that the occurrence of secondary embryogenesis in anther culture is such that the same microspore can give rise to more than one embryo; otherwise, genetic segregation would have been expected to result in a highly heterogeneous array of anther-derived plants, precluding the occurrence of genetically identical individuals. The phenomenon of secondary embryogenesis has two significant impacts: the first is that a population of anther-derived plants cannot be used to examine segregation analysis until the duplicate plants have been eliminated from the population because of the skewness that will result from sampling the same genotypes repeatedly; the second is that one of the measures of androgenic response, i.e., frequency of embryos per culture unit, may differ qualitatively among clones. One clone may exhibit a greater induction rate among microspores whereas another with an equivalent embryo yield may represent fewer induced microspores that have given rise to numerous secondary embryos. The first would be preferable because, in most situations, the objective is to regenerate as large a sample of the microspore population as possible. Examination of qualitative or quantitative traits without elimination of duplicates by molecular analysis would lead to erroneous conclusions.

Distorted segregation ratios among DH progenies favouring alleles of the more anther culture responsive parent have been found in various crops including pepper, *Capsicum annuum* (Lefebvre *et al.*, 1995), broccoli, *Brassica oleracea* (Orton and Browers, 1985), ryegrass, *Lolinum perenne* (Hayward *et al.*, 1990), rice, *Oryza sativa* (Guiderdoni, 1991), barley, *Hordeum vulgare* (Graner *et al.*, 1991; Heun *et al.*, 1991; Zivy *et al.*, 1992), maize, *Zea mays* (Bentolila *et al.*, 1992; Wan *et al.*, 1992; Murigneux *et al.*, 1993a) and rapeseed, *Brassica napus* (Tanhuanpää *et al.*, 1994; Cloutier *et al.*, 1995). Such studies have led to the identification of chromosomal regions associated with various aspects of microspore or anther culture responsiveness.

Even though Schön *et al.* (1991) found preferential transmission of isozyme alleles characteristic of one parent to the DH progeny derived from a barley F_1 by the *Hordeum bulbosum* technique, the recombination distances between known linked genes did not differ from those estimated with a control F_2 population. A similar result was found for *Brassica napus*, where deviations from Mendelian segregation were observed in both a DH and an F_2 population (Ferreira *et al.*, 1994). However, no association of alleles to microspore responsiveness could be made. Thiagarajah and Stringam (1993) found that genes controlling seed colour and leaf hairiness in *Brassica juncea* segregated similarly in backcross, F_2 and microspore-derived populations. Suenaga and Nakajima (1993a) compared the

segregation of eight genetic markers in F_2 and DH lines of wheat, using inter-generic crosses with maize followed by embryo culture to derive the DH population. The general lack of distorted segregation for the markers was taken as evidence of an absence of correlation between the markers and efficiency of embryo formation, which differed between the two parents of the F_1 hybrid used to derive both populations.

Rivard *et al.* (1996) constructed genetic maps comprising 84 RFLP loci of the self-incompatible potato species, *Solanum chacoense*, using anther-derived, selfed (after bud pollination), and hybrid progenies from selections in two accessions. Ten per cent of markers exhibited skewed segregation in the hybrid population, compared to 30% in the selfed population, and 46% and 70% in the two anther-derived populations. A major difference was that most of the skewed markers in the selfed population occurred in the proximity of the *S*-locus whereas they were scattered throughout the genome in the anther-derived populations. A differential recombination rate between male and female gametes was suggested as an underlying reason for the longer maps generated by anther-derived populations. The consequences of segregation distortion must be considered before attempting to assign linkage groups (Foisset and Delourme, 1996).

Devaux *et al.* (1993) examined RFLP and RAPD profiles of 30 anther-derived and 30 *H. bulbosum*-derived DHs of barley in comparison with seed-derived lines of the inbred barley cultivar, Igri. No polymorphism was detected; however, when DNA was digested with the methylation-sensitive restriction enzyme *Hpa*II, RFLP variation was detected primarily in the anther-derived lines, suggesting that DNA methylation changes had occurred during DH production. In a study of two anther-derived generations of wheat, Rode *et al.* (1987) did not detect differences in the extent of methylation of ribosomal DNA compared to the parental line. Instead, variation was found in the organization of the non-transcribed spacer region of rDNA in the first cycle, a difference that persisted but did not change through the second cycle of anther culture.

Agronomic Performance of Doubled Haploid Lines

Various comparisons have been made of the agronomic performance of doubled haploids and sexually derived inbred lines to address the concerns first reported by Arcia *et al.* (1978), Schnell *et al.* (1980) and Brown and Wernsman (1982) on the agronomic inferiority of anther-derived DHs of tobacco. The conclusions vary among crops and germplasm examined.

Murigneux *et al.* (1993b) compared 81 F_6 SSD lines of maize with 100 DHs derived by anther culture of the same F_1, and concluded that the different reproductive systems had no influence on the reassortment of the genetic variability of polygenic traits. However, the opportunities for additional recombination to occur over several sexual cycles in the SSD population resulted in more frequent recombinations when genetic distances between loci were close. The implication was that more DH than SSD individuals need to be scored to obtain recombination

between closely linked genes. In order to increase opportunities for recombination in an F_1 destined for androgenesis, De Buyser *et al.* (1985) recommended two generations of selfing prior to extraction of haploids. Iyamabo and Hayes (1995), however, found only a modest effect of generating more favourable genotypes by allowing an additional round of recombination, and concluded that such marginal advantage should be weighed against the delay of deriving an additional generation. Maternal DHs of maize obtained by crossing with haploid-inducing pollinators were compared to SSD lines and found to represent a random gametic array (Lashermes *et al.*, 1988).

Kubba *et al.* (1989) compared SSD with DH lines of brussel sprouts (*Brassica oleracea* var. *gemmifera*) over two seasons. There was less variation among plants within the DH lines compared to the SSD lines. This finding was taken as evidence of greater homozygosity within the DH lines. For the eight horticultural characteristics examined, only one exhibited a difference between the two populations, however; and this difference was for waste weight, i.e., the partitioning of total yield into marketable and waste fractions. The difference may have been due to sampling variation that occurred as a result of selection of different double cross hybrids as starting material for the two populations or to true gametoclonal variation.

In a comparison of seven agronomic characters between DH and SSD lines of two wheat cultivars ('Kitt' and 'Chris'), Baenziger *et al.* (1989) found three significant differences within both the 'Kitt' and 'Chris' families; however, the two families did not differ for the same three of seven characters measured. In general, when a significant difference was found, the DH lines were agronomically inferior to the SSD lines. For most characters, there was greater heterogeneity among the DH lines than among the SSD lines, a finding that was taken as evidence that the variation was due more to gametoclonal variation than to cultivar heterogeneity. The authors concluded that gametoclonal variation should not greatly hinder a breeding programme because the lower-yielding lines could easily be culled. A similar conclusion was reached by Mitchell *et al.* (1992) in a comparison of SSD and anther-derived lines from an F_1 hybrid of wheat, where the mean yield of SSD lines exceeded that of anther-derived lines in one of the two seasons but the highest-yielding 20% of lines from each method did not differ. Many of the lower-yielding lines examined by De Buyser *et al.* (1985) were found to exhibit cytological abnormalities.

Marburger and Jauhar (1989) examined variation among and within DH lines of wheat cv. Chris in comparison with the source cultivar. All entries in their study were two sexual cycles post-tissue culture to eliminate differences due to epigenetic variation. The DHs averaged significantly lower 1000 kernel weight and yield per tiller than the source cultivar. In addition to variation among DH lines for all five agronomic traits examined, cytogenetic and isozyme variation was also detected. Most of the DHs that differed significantly from 'Chris' were inferior agronomically. Differences between this and the study by Baenziger *et al.* (1989) may have been due to different field plot techniques; more space between adjacent plants and shorter rows characterized the Marburger and Jauhar (1989)

study. Because colchicine-doubled haploids did not differ consistently from spontaneously doubled haploids, the authors concluded that the variation was gametoclonal rather than induced during the doubling phase. Likewise, Kumashiro and Oinuma (1986) dismissed colchicine treatment as the source of gametoclonal variation in a comparison between colchicine-doubled and spontaneously doubled haploids of tobacco. Wan and Widholm (1995) doubled anther-derived haploids of maize with colchicine, pronamide, or amiprophos-methyl without increasing the mutation rate over that of tissue culture-derived maize that had not received any treatment with antimicrotubule agents. They also concluded that these agents were innocuous with regard to generating gametoclonal variation. In contrast, Suenaga and Nakajima (1993b) attributed the gametoclonal variation observed among DH lines of wheat derived from wheat × maize crosses to the colchicine treatment applied to primary DHs. Some DH lines exceeded the mean agronomic performance of the source cultivar, but most that differed significantly were inferior.

Skinnes and Bjørnstad (1995) examined DH and SSD lines derived from six F_1 hybrids of wheat over 2 years. Various inferiorities of DHs were found; however, they differed among crosses, characters and years. The frequency of SSD and DH lines that exceeded the grain yield of the superior parent of the hybrid used to derive them also differed among crosses such that SSD lines prevailed in two crosses whereas DH lines prevailed in a third cross.

In barley, Powell *et al.* (1986a) compared the agronomic performance of anther-derived DHs with maternally derived DHs using the *Hordeum bulbosum* technique and SSD lines, all from F_1 hybrid material. The anther-derived DHs were inferior to the other two sources of inbreds for five of the seven agronomic traits. In another comparison between *H. bulbosum*-derived DHs and SSD lines, Caligari *et al.* (1987) found little evidence for reduced vigour in the DH population for eight of the nine agronomic characters; only for 1000-grain weight and height in one of the five families was the DH mean significantly lower than the corresponding SSD mean. In a further study, Powell *et al.* (1986b) hypothesized that the difference observed for 1000-grain weight was unlikely to be due to microspore selection, but rather due to an excess of coupling linkages between loci with negative epistasis expressed in the DH population, implying an effect of reduced recombination. Bjørnstad *et al.* (1993) derived DHs by anther culture and *H. bulbosum* from three barley crosses and compared the agronomic performance with SSD lines. Inconsistent differences were found among the methods for the three crosses. The authors concluded that the methods were equivalent, although not identical, and that there was no consistent negative impact of anther culture despite the inferior performance of some anther-derived lines. The *H. bulbosum* technique was also used to derive two generations of maternal doubled haploids (DH/DHs) in both barley and wheat to examine the occurrence of gametoclonal variation (Snape *et al.*, 1988). For barley, there was no variation observed among DH/DH lines derived from single DHs for all the seven characters examined in 10 DHs derived from two different cultivars, indicating an absence of gametoclonal variation. This corroborated the findings of Finnie *et al.* (1991) that there

was little cytological, biochemical or molecular evidence of gametoclonal variation among microspore-derived DHs of barley. For two of the three wheat genotypes investigated by Snape *et al.* (1988), however, gametoclonal variation was observed for several characters, always in the direction of poorer agronomic performance than the source cultivar. Snape *et al.* (1988) examined maternal DHs derived from maternal DHs of two wheat cultivars; detrimental gametoclonal variation in the form of later maturity, lower yield and reduced grain weight per ear was reported. The differences were attributed to random mutation, and the authors suggested a similarity to gametoclonal variation due to anther culture. The relative tolerance of a polyploid genome, such as wheat compared to a diploid genome such as barley, to genetic change has been cited as a cause for the difference between species in the documentation of gametoclonal variation (Finnie *et al.*, 1991).

Comparisons between anther-derived and control lines of rice (*Oryza sativa* L.) have also indicated the occurrence of gametoclonal variation (Sathish *et al.*, 1995). The progenies of anther-derived calluses were generally shorter, with fewer tillers and later maturing than control plants that were seed-propagated from the same cultivar used for anther culture. The indirect androgenesis typical of rice, i.e., passage through a callus phase before regeneration of haploids or DHs, makes the occurrence of gametoclonal variation even more likely than for crops that express direct androgenesis. From 43 original lines, N'Guessan *et al.* (1994) selected two anther-derived rice lines that exhibited greater tolerance to the rice water weevil (*Lissorhoptros oryzophilus*) than the anther donor. Although, these lines displayed a decrease in root damage in response to infestation over 2 years, poor plant characteristics of these lines with regard to other agronomic traits prevented their direct utilization in cultivar release.

A variety of causes has been hypothesized to underlie the frequently reported yield reduction of tobacco anther-derived lines (Reed, 1996). Nielsen and Collins (1989) compared the agronomic performance of gynogenic (derived by wide crosses with *Nicotiana africana*) and androgenic DHs with selfed lines of a burley tobacco cultivar. Means of most traits did not differ among the three populations. However, yield reduction of more than 18% was observed for one gynogenic and one androgenic line. Reduced performance of these lines with respect to other characters was also found. Yung and Wernsman (1990) observed a 4.5% yield reduction in a comparison of 64 randomly selected anther-derived DHs with the source cultivar. However, by using a recurrent selection scheme within the DH population, yield comparable to the source cultivar had been recovered in the third cycle. Significant additive genetic variance for the maternal components contrasted sharply with insignificant male components for plant height and yield. This was thought to reflect the relative tolerance of the female gametophyte to genetic abnormalities compared with the male gametophyte. They, therefore, questioned the ability to maintain gametoclonal variation in a random mated anther-derived population. The role and consequences of differential DNA replication in vigour reduction and cytological abnormalities of tobacco DHs, first reported by De Paepe *et al.* (1981) have recently been reviewed by Reed (1996). In general, there has not been a linear relationship between the amount of DNA

amplification and loss in vigour, but some of the poorest tobacco DHs expressed the greatest increase of DNA content. Performance may have more to do with which genetic sequences have been amplified rather than total DNA *per se*.

Fewer studies have been conducted on gametoclonal variation in cross-pollinated crops because of the expected variation due to genetic recombination superimposed on culture-induced variation. In a study of anther-derived plants of *Populus maximowiczii*, Stoehr and Zsuffa (1990) concluded that selection during the formation of plants from microspores reduced the array of alleles among the anther-derived plants. They considered that most of the variation among haploid lines was due to genetic differences among gametes. Sarvesh *et al.* (1994) reported androclonal variation in the form of dwarf and large flower head types among anther-derived regenerants of the oilseed crop, *Guizotia abyssinica* Cass., also known as niger. The control population consisted of seedlings, presumably generated from intercrosses within the genetically diverse cultivar. Due to its self-incompatible nature, niger variants could have arisen from exposure of recessive alleles present in the parent cultivar.

Selection for Specific Traits during Haploidization – 'Hybrid Sorting'

Recovery of agronomically useful gametoclones has been attempted both with and without specific selection agents during the haploidization process. Lashermes (1991) has outlined the considerations for imposing *in-vitro* selection on microspore culture systems. Szarejko *et al.* (1991) discussed the integration of mutation breeding with androgenesis. Witherspoon *et al.* (1991) examined 545 anther-derived haploids of a potato virus Y susceptible cultivar of tobacco without selective agents during the haploidization process. A single haploid plant survived inoculation with a virulent strain of the virus. The chromosome number of the variant was doubled, and the selfed progeny proved to be equally resistant to the virus. Other than the PVY resistance that was found to be controlled by a single gene, the gametoclonal variant also exhibited an 18% yield reduction over the parental cultivar. In an additional study of this variant, Yung *et al.* (1991) found that the yield reduction was not due to the resistance gene and that much of the yield reduction could be recovered after backcrossing the variant with the cultivar from which it had been derived.

Resistance to tobacco black shank disease (*Phytophthora parasitica*) was recovered among anther-derived DHs from susceptible parental cultivars of tobacco without the use of a selective agent during haploidization (Nichols and Rufty, 1992). In this study, 75 anther-derived and 75 SSD lines were screened for resistance, among which eight anther-derived DHs were found with stable resistance. No stable resistance was found among the SSD lines.

Fadel and Wenzel (1993) selected 375 green lines from 242 000 anthers of wheat hybrids between parents resistant and susceptible to *Fusarium* head blight by incubating anthers in the presence of various concentrations of fungal toxins. Leaf bioassays of the *in-vitro* selections were consistent with resistant reactions.

The selection was presumed to be for gene complexes associated with resistance that were derived from the resistant parents rather than for new variation. However, the two potential sources of variation could not be distinguished.

By combining mutagenesis treatments with *in-vitro* selection in the presence of chlorsulphuron, Swanson *et al.* (1988) recovered two chlorsulphuron tolerant mutants in *Brassica napus* microspore cultures. Such cultures would have been expected to produce tens of thousands of embryos in the absence of selection. Self-pollination of the colchicine or spontaneously doubled mutants revealed a semi-dominant mode of inheritance of the resistance controlled by a single gene. A major advantage of the microspore culture system was that selection occurred at the haploid level, resulting in homozygous mutant genotypes after chromosome doubling.

Ye *et al.* (1987) cultured anthers of a saline-susceptible and a saline-resistant barley cultivar and their F_1 hybrid on media with 0.4–0.8% Na_2SO_4. Microspore-derived lines were considered salt tolerant if selfed seed of spontaneously doubled haploids germinated under salt stress (3.25–5% Na_2SO_4). Of 37 lines derived from the F_1 hybrid, nine were salt tolerant (seven from salt-free treatment and two from salt stress imposed during anther culture). The elevated salt tolerance of these nine lines was attributed to genetic recombination rather than gene mutation. Microspore-derived lines of the susceptible cultivar were uniformly susceptible. The salt stress imposed during anther culture eliminated all susceptible genotypes but may have also prevented the regeneration of some tolerant lines because the control treatment resulted in greater recovery of salt tolerant lines.

Bozorgipour and Snape (1992) found that the vigour of anther-derived haploid embryos, as measured by the coleoptile height in culture, could be used to predict the agronomic performance of DHs of barley in the field. Of the many correlations attempted between *in-vitro* and *in-vivo* growth, this was the only one that was consistently positive across three hybrid populations; the regression equation was statistically significant in two populations and nearly significant ($p = 0.054$) in the third population. These results suggest that the least valuable genotypes can be eliminated *in vitro* so that field evaluations can be limited to only the most vigorous embryos.

In rapeseed (*Brassica napus*), elevated levels of erucic acid in the seed are sought by the oleochemical industry. Albrecht *et al.* (1995) found that the erucic acid level could be determined in a single cotyledon of microspore-derived embryos while the remaining embryo could be used to generate an *in-vivo* plant. Oil quality of the embryo cotyledons was highly correlated with that of the seeds derived from the regenerated plants. This allows the opportunity for early selection of high erucic acid embryos and disposal of the majority of undesirable embryos.

Conclusions

Gametoclonal variation has been documented in a wide variety of crop plants. Like all sources of variation in highly selected species, the effects are likely to be deleterious on agronomic performance. Some documented gametoclonal variation

may actually be epigenetic as a result of DNA methylation during tissue culture, and may subside after sexual cycles. Other variants have been attributed to single gene mutations and hence persist. Directing gametoclonal variation by imposing selection agents on *in-vitro* processes has met with some success and offers a better alternative to other *in-vitro* selection schemes because of the haploid nature of microspores and the diverse genetic array of microspores from a cross-pollinating species or F_1 hybrid. It would appear that gametoclonal variation is less of a hindrance and more of an opportunity in plant breeding efforts.

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8. Protoclonal Variation in Crop Improvement

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Introduction

Protoplasts do not have cell wall, hence they are suitable material for introducing foreign genes in plants by genetic engineering and somatic hybridization of interspecific and intergeneric plants. Protoplasts can be isolated from diverse tissues in a wide range of species, and they are ideally suited for research in somatic cell genetics. In addition, investigations on the processes of plant regeneration and their control may contribute to a better understanding of ontogenic processes in plants. In 1971, Takebe and his collaborators isolated protoplasts from leaves in *Nicotiana* which could be divided in culture and ultimately regenerated into the whole plants (Takebe *et al.*, 1971). This report, along with Carlson's (1973) work on protoplast-derived plants in *Nicotiana* resistant to the wildfire disease, and Shepard *et al.*'s (1980) findings on *Solanum tuberosum* protoclones are evidence to support the role of protoplast cultures in the genetic improvement of crops. Plant regeneration from protoplasts has been achieved in more than 300 species. Progenies of protoclones can show considerable protoclonal variation (Jain and Newton, 1989). Molecular studies have provided evidence of the induction of genetic changes in protoclones through the changes of DNA sequences during protoplast culture (Rose *et al.*, 1986; Kane *et al.*, 1992). Protoclonal variation among regenerated plants is ubiquitous, and some of the specific mutations have the potential to be used in plant improvement.

Protoclonal Variation and its Genetic Response

Protoclonal variation offers the advantage of selection from vast populations of cells and regenerated plants derived from a single cell. Therefore, it is important to have efficient plant regeneration from protoplasts of major crop plants independently of genotypes. Protoclonal variation could arise due to several factors, and plants regenerated from protoplasts can show extensive changes, including alterations in gross morphology, maturity, yield and disease response (Shepard *et al.*, 1980; Jain and Newton, 1989).

Changes in Morphological Traits

Changes in morphological traits have been reported in the progeny of protoclones of *Nicotiana* and other crop species (Table 1). In *Hyoscyamus muticus*, gigantic variants of the R2 progeny had a greater percentage of alkaloid content than control

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Table 1. Protoclonal variants with morphological traits

Species	Traits	Reference
Brassica juncea	(+) pod number, (+) seed yield	Eapen *et al.*, 1989
Brassica napus	(#) seed weight, (+) herbicide resistance, (#) maturing, seed colour	Jain and Newton, 1988; Jain and Newton, 1989
Hyoscyamus muticus	(#) maturing, (+) plant yield	Giri and Ahuja, 1994
Nicotiana sylvestris	(–) plant size (dwarf)	Prat, 1983
Nicotiana tabacum	(+) resistant to bacterial wilt	Daub and Jenns, 1989
Oryza sativa	(#) culm length, (–) ear length, (+) grain yield, (+) panicle number, (–) spikelets/panicle, (+) stem/plant	Ogura *et al.*, 1989

(+): increase in the character; (–): decrease in the character; (#) changes in both directions.

plants. Although they resembled tetraploids, cytologically these plants were confirmed to be diploids. This gigantic growth habit trait was inherited for more than three successive generations when evaluated in field trials (Giri and Ahuja, 1994). In *Nicotiana tabacum*, progeny analyses in protoclones revealed that significant variation was found in resistance to black shank and bacterial wilt, two diseases for which the parent cultivars have low levels of resistance. In *Brassica juncea* the progeny of the protoclones was evaluated for various agronomic characteristics (Eapen *et al.*, 1989). Some of the progeny plants varied in height and time required for flowering. Three progenies of protoclones flowered after 31–34 days, compared with 36–43 days for the parent plants. Significantly higher pod numbers were obtained in five progenies of protoclones but, of these, one plant had higher seed yields than the parent plants. In *Nicotiana sylvestris*, Prat (1983) revealed that eight of 13 progenies of protoclones produced phenotypes not observed in the parent plants, that is, dwarf, abnormal leaf shapes. In *Oryza sativa*, the progeny of protoclones showed about 25% semi-lethal segregants among their first progenies in cv. Fujisaka 5. The culm length of cv. Nipponbare protoclones was uniform and slightly shorter than the control, whereas the culm length was slightly longer in protoclones of cv. Iwaimochi. The viable protoclones of cv. Fujisaka 5 exhibited a considerably shorter culm as compared with the control (Ogura *et al.*, 1989). Jain and Newton (1988) reported variation in total seed weight per plant of self-pollinated *Brassica napus* protoclones in the first progeny of regenerated plants. These results indicated that some of the protoclonal variation had been inherited by the subsequent generation which should be called mutation.

Chromosomal Alteration

Structural alterations and changes of ploidy in chromosomes have been observed during protoplast culture in many species. These findings created considerable in-

terest in the possibility of use in plant breeding and of the interpretation of the nature of mutations.

Chromosomal alteration may be generated through several types of nuclear chromosomal rearrangements and losses, gene amplification or deamplification, or non-reciprocal mitotic recombination events. Recent reports concerning chromosomal alteration of protoclones are summarized in Table 2. Kanda *et al.* (1988) found polyploids and aneuploids in protoplast cultures of *Oryza sativa* cv. Norin 8. The protoclones showed a high percentage of tetraploids, and only 30% of protoclones were diploid. In *Solanum tuberosum*, of over 200 plants screened, 57% had 48 chromosomes, and the remaining plants were aneuploids (2n = 73–96) (Karp *et al.*, 1982). A high frequency of chromosome doubling was also observed in *Solanum tuberosum* plants regenerated from dihaploid protoplasts (Wenzel *et al.*, 1979). When monohaploid (2n = x = 12) leaves were cultured, regenerated shoots doubled in chromosome number. Karp *et al.* (1982) reported that when leaves from the doubled monohaploids were cultured, half of the shoots regenerated from them remained at the doubled monohaploid level (2n = 4 x = 48). Similarly, regeneration from cultured dihaploid leaves gave approximately 50–60% tetraploid plants (Jacobsen, 1981). These results do not explain all the aspects of genetic changes of protoclones. Changes at the level of DNA sequences cannot be readily detected by cytological analysis, but could be characterized by genetic and/or molecular analyses.

Molecular Analysis of Genetic Changes in Protoclones

Major advances in molecular biology have been made, including gene cloning strategy and polymerase chain reaction (PCR) for the isolation of several functional genes and repetitive DNA sequences. These techniques have been applied for the analysis of DNA rearrangements during protoplast culture. Restriction fragment length polymorphisms (RFLPs) and randomly amplified polymorphic DNAs (RAPDs) using PCR can estimate the changes of DNA sequences between

Table 2. Chromosomal alteration in protoclones

Species	Change	Reference
Actinidia deliciosa	Chromosome number	Cai *et al.*, 1991
Asparagus officinalis	Aneuploidy, polyploidy	May and Sink, 1995
Brassica oleracea	Polyploidy	Cardi and Earle, 1995
Fescuta arundinacea	Chromosome number	Humphreys and Dalton, 1991
Hordeum vulgare	Aneuploidy, polyploidy	Wang *et al.*, 1992
Lotus corniculatus	Polyploidy, structural change	Niizeki *et al.*, 1990
Nicotiana tabacum	Aneuploidy	Vyskot *et al.*, 1991
Oryza sativa	Polyploidy	Guiderdoni and Chair, 1992
Petunia hybrida	Polyploidy	Lewis-Smith *et al.*, 1990; Oh *et al.*, 1995
	Aneuploidy	Lewis-Smith *et al.*, 1990
Saintpaulia ionantha	Polyploidy	Winkelmann and Grunewaldt, 1995

protoclones and their parent plants. Recently, several novel cloning techniques for specific DNA fragments have been proposed, such as genomic subtraction (Yokota and Oishi, 1990). Indeed, in an analysis of DNA changes during protoplast culture, genomic subtraction was performed and resulted in the isolation of several DNA fragments (Fukuoka *et al.*, 1994). Analyses of DNA changes during protoplast culture are summarized in Table 3. Since Landsmann and Uhrig (1985) first reported the characterization of genetic changes of protoclones by Southern hybridization analyses in *Solanum tuberosum*, using rDNA and hypervariable sequences as probes, several reports have appeared on nuclear and mitochondrial DNA rearrangement during protoplast culture. Recently, the molecular mechanism of DNA rearrangement in proto-calli was analysed in *Oryza sativa* chloroplast (plastid) DNA (Kawata *et al.*, 1995, 1997). It was demonstrated that short inverted repeats function as hotspots of intermolecular recombination, giving rise to oligomers of deleted plastid DNAs.

Change in Mitochondrial DNA

The mitochondrial DNA (mtDNA) of higher plants, known to be large and heterogeneous (Ward *et al.*, 1981), can be generally considered as a master molecule containing all the sequence information. In most cases, mtDNA is characterized by the presence of repeated sequences (Lonsdale *et al.*, 1984). Since mtDNA contains varieties of direct or inverted repeats which tend to permit homologous recombination, mtDNA consists of molecules of different sizes which interconvert via recombination, mainly involving repetitive sequences. The size and number of such repeats vary among species. The greater the number of the repeats, the more complex is genome structure (Lonsdale *et al.*, 1984) with a mixture of circular and/or linear molecules (Bendich and Smith, 1990). RFLP analyses using the probes as mitochondrial DNA fragments were performed in several crops, revealing both an increase and a decrease in copy number and DNA rearrangements during protoplast culture. In *Nicotiana sylvestris*, novel mtDNA organization of protoclones was due to the amplification of recombinant substoichiometric DNA sequences that pre-exist in the parent plant (Vitart *et al.*, 1992). Li *et al.* (1988) reported that the progenies of each regenerated plant examined possessed different mtDNA restriction patterns. In *Sorghum bicolor* (Kane *et al.*, 1992) and *Medicago sativa* (Rose *et al.*, 1986), DNA polymorphism in mtDNA was also noted. Fukuoka *et al.* (1994) observed, that the sequence analysis of the protoclones suggested that most of the clones originated from mtDNA. Comparison of copy number fluctuation patterns of the mitochondrial functional genes with that of the clones suggested multiformity and/or construction-specific amplification of mtDNA. These results suggest that changes in the molecular organization of mtDNA, are induced during protoplast culture, are associated with protoclonal variation.

Change in Plastid DNA

Although plastid DNA (ptDNA) is considered to be more stable than other genomes, because of the compact size and non-telomeric (circular) structure, it

Table 3. DNA changes in protoclones

Species	Genome	Change	Reference
Brassica campestris	mtDNA	Inversion, duplication	Shirzadegan *et al.*, 1991
Festuca pratensis	nuDNA, mtDNA, cpDNA	Polymorphism	Valles *et al.*, 1993
Lolium multiflorum	(RAPD)	Polymorphism	Wang *et al.*, 1993
Medicago sativa	mtDNA, cpDNA	Polymorphism	Rose *et al.*, 1986
Nicotiana rustica	nuDNA	Change of t-DNA structure	Furze *et al.*, 1987
Nicotiana sylvestris	nuDNA, mtDNA	Polymorphism, deletion	Vitart *et al.*, 1992; Li *et al.*, 1988
Oryza sativa	mtDNA, cpDNA	Change of copy number	Fukuoka *et al.*, 1994
	cpDNA	Deletion, recombination	Kawata *et al.*, 1995
	nuDNA	Polymorphism	Brown *et al.*, 1990
Scilla siberica	nuDNA	Change of copy number	Deumling and Clermont, 1989
Solanum tuberosum	nuDNA	Polymorphism	Landsmann and Uhrig, 1985
Sorghum vulgare	mtDNA	Polymorphism	Kane *et al.*, 1992

nu: Nuclear, mt: mitochondrial, cp: chloroplast.

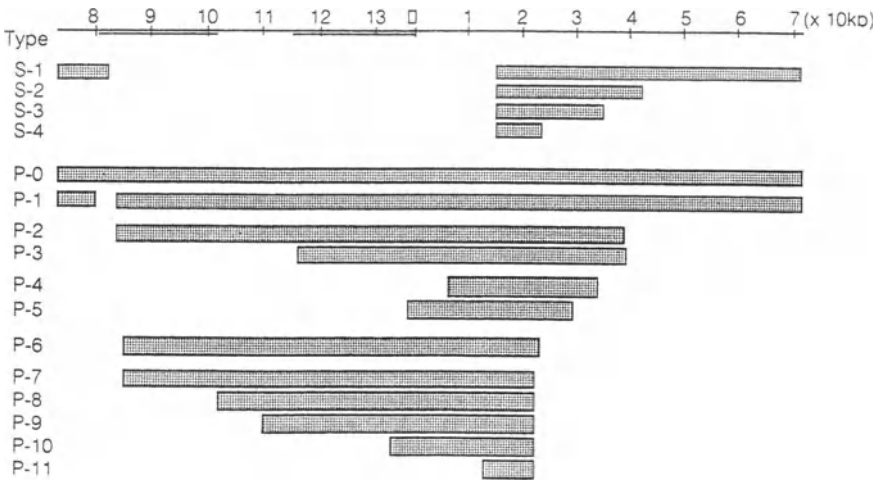

Figure 1. Schematic diagram of pt DNA retained in different protoclones in rice. Type 'S' series are cell lines from seed-derived protoplasts, and type 'P' series are cell lines from pollen-derived protoplasts. The unit scale represents 10 kb. The double line shows inverted repeats.

is susceptible to recombination under specific environmental conditions. Various series of recombinations of ptDNAs have occurred through insertion/deletion, inversion, recombination and transposition in higher plants. Crouse *et al.* (1986) suggested that the short repeats may serve as substrates for the recombination systems. Each plastid contains many copies of its genome, thus potentially allowing intermolecular recombination of individual DNA molecules to occur.

Kawata *et al.* (1995) observed changes in ptDNA using protoclones in *Oryza sativa* derived from protoplasts. They found that ptDNA in *Oryza sativa* underwent deletions (Fig. 1). The deleted ptDNA of proto-callus was organized as a head-to-head or tail-to-tail structure (Fig. 2). In order to elucidate the fundamental molecular mechanism of the structural alteration of ptDNA and to characterize the mechanism of ptDNA recombination of protoclones, the DNA structure around the junction point of deleted ptDNAs, the region which contained the expected junction point of the cell line, was cloned and sequenced (Fig. 3) (Kawata *et al.*, 1997). It was notable that these jointed inverted repeats were interposed by a sequence which directly continued to either of the jointed inverted repeats (extra sequence). From the results described above, a model of intermolecular recombination between the ptDNA molecules mediated by a short inverted repeat was proposed (Fig. 4). This model can explain the experimental results, i.e., large-scale deletion of ptDNA and direct fusion of deleted ptDNAs in inverted orientation, which is linked by continuous extra sequences derived from either of the joined ptDNA molecules. The model further takes into account the retention of functionally essential regions of ptDNA even after several processes of intermolecular recombinations, mediated by short inverted repeats found in ptDNA.

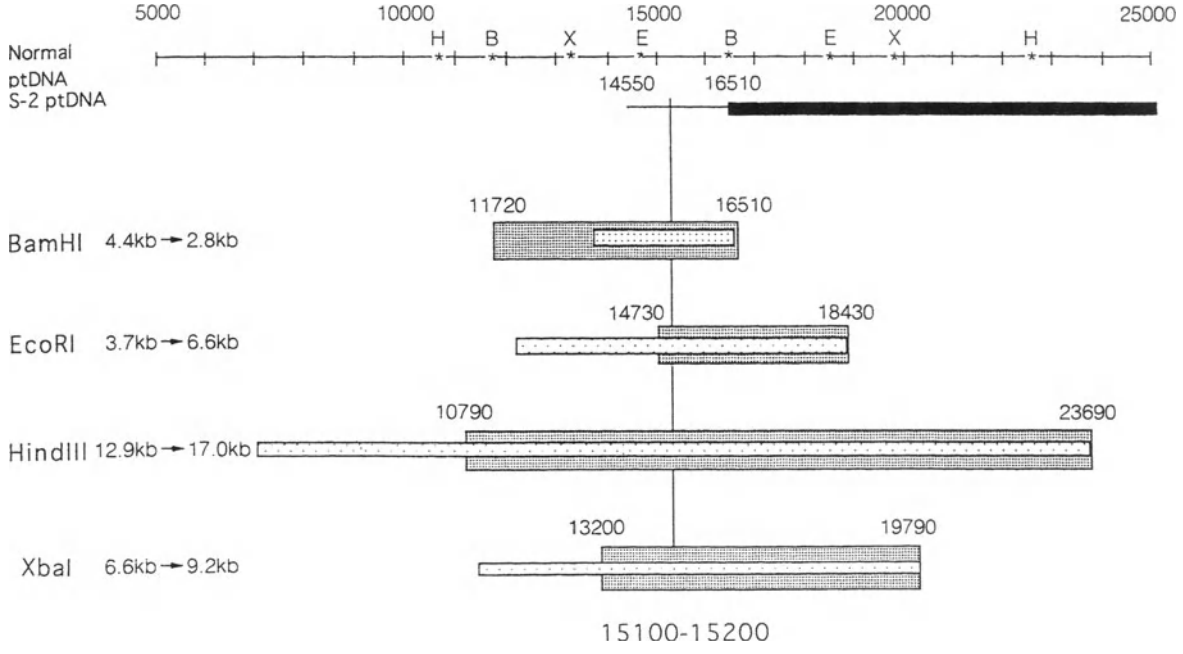

Figure 2. Restriction map of the rice protoclone 'S-2'. The thick solid bar represents a region identical to the corresponding region of normal pt DNA. The heavily dotted boxes represent the hybrid fragments expected from the sequencing data, whereas the thinly dotted boxes represent the retained regions. Restriction sites are indicated by the initials of the respective enzymes. The predicted and observed sizes of each restriction fragment are shown on the left.

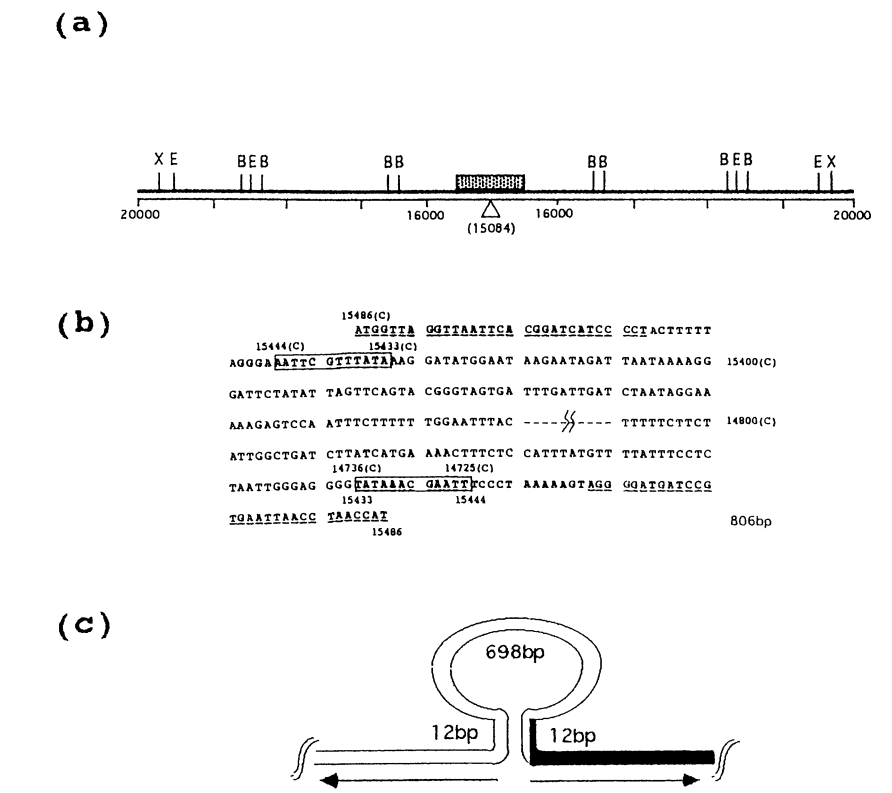

Figure 3. **(a)** Physical map at one of the telomeric regions of the deleted pt DNAs from a protoplast-derived cell line. Subcloned fragments for further analyses are in shaded boxes. Numbers indicate the positions of rice chloroplast DNA sequence (Hiratsuka *et al.*, 1989). Triangles show the expected junction points of deleted pt DNAs of each cell line. B, D, E, H, K and X depict the restriction sites of *Bam*HI, *Dra*I, *Eco*RI, *Hind*III, *Kpn*I and *Xba*I. **(b)** Nucleotide sequences around the junction points of deleted pt DNAs from a protoplast-derived cell line. Bold capitals indicate the sequences formed as inverted repeats based on the tail-to-tail configuration of deleted pt DNAs. The intermediate sequence, which is derived from either one of the deleted pt DNA molecules, is flanked by short inverted repeats, which are depicted as boxed sequences. Underlined nucleotides are *Bam*HI sites. One of the short inverted repeats, which are both underlined and boxed, corresponded to the junction points of deleted pt DNAs. **(c)** Structure of the pt DNAs of a protoplast-derived cell line around the junction points of deleted pt DNAs. Joined pt DNA molecules are shown as white or black boxes. Arrowheads indicate the short inverted repeats. Dashed region flanked by the short inverted repeats shows the sequences derived from the white boxed pt DNAs. One of the inverted repeats, corresponding to the junction point, is shown by solid black.

These results suggest that short inverted repeats function as hotspots of intermolecular recombination of ptDNAs of protoclones.

Change in Nuclear DNA

Besides gross chromosomal aberrations, protoclonal variability covers a wide range of genetic changes, such as alteration of nuclear DNA sequence of func-

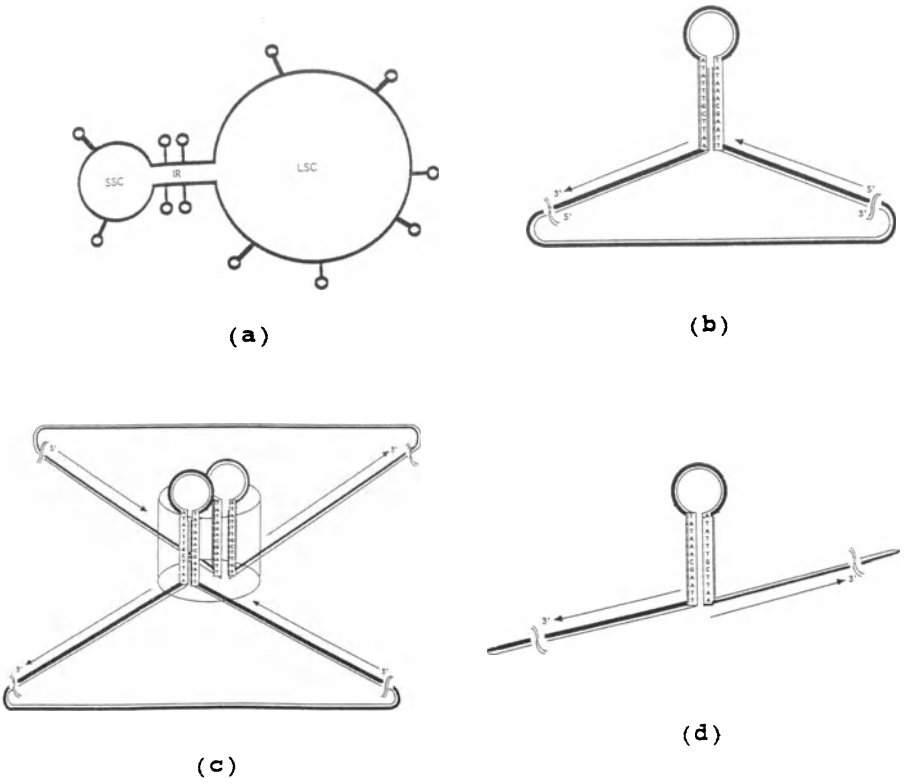

Figure 4. Possible intermolecular recombinations via short inverted repeats giving rise to an oligomer possessing head-to-head or tail-to-tail configuration of deleted pt DNAs. **(a)** The varieties of short inverted repeats are included in the single circular pt DNA molecule. SSC: small single copy, LSC: large single copy. **(b)** One of the regions corresponding to the short inverted repeat, which is demonstrated in the DNA rearrangement of a protoplast-derived cell line. **(c)** Intermolecular recombination event between pt DNAs occurs via homologous short inverted repeats. **(d)** New DNA molecules with extra sequences flanked by short inverted repeats are generated by fusion of deleted pt DNA molecules.

tional gene (Brown *et al.*, 1990). In addition to such changes, other classes of genetic variability may arise during protoplast culture including modification in repetitive DNA.

In *Scilla siberica*, Deumling and Clermont (1989) reported the loss of about 80% of the initial nuclear DNA content and 95% of the satellite sequences in protoclones. Sequences hybridizing with certain protein-coding genes were markedly reduced in copy number, whereas the proportion of rDNA was increased. In Southern-blot analyses with a probe consisting of the central part of 25S rDNA, rearrangement could be detected among tuber-derivatives of protoclones. Wang *et al.* (1993) reported DNA alteration of protoclones in *Lolium* species by RAPD analyses. In *Oryza sativa* protoclones, Brown *et al.* (1990) showed a significant increase in DNA polymorphism as compared with control seed-derived plants. Analysis of gene sequences representing different functional

domains revealed that such polymorphisms are apparently widespread without any association with a particular region. Landsmann and Uhrig (1985) reported the deficiency of ribosomal RNA genes in protoplasts of *Solanum tuberosum*. In *Nicotiana rustica*, Furge *et al.* (1987) reported the changes of t-DNA structure in protoclones compared to parent plants. These reports indicated the possibility that drastic changes in phenotype had been induced by the rearrangements of functional nuclear genes.

Application of Protoclonal Variation for Crop Improvement

During the past 25 years, it has become possible to regenerate plantlets from explants and/or callus. Though protoclonal variations occur as a result of mutations in somatic cells, it is not yet proven if these mutations occur during protoplast culture. Carlson (1973) proposed the advantage of the application of mutagens in cells for plant breeding strategy. Oono *et al.* (1984) proposed the importance of single-cell systems such as the single pollen-derived cell and protoplast culture for the analysis of mutations during somatic cell culture. Since then, it has been demonstrated by molecular analysis that somatic mutation was induced during somatic cell culture (Peschke *et al.*, 1987, Muller *et al.*, 1990). Thus, somatic mutations induced in tissue culture will be expected to establish useful agronomic variants with great potential in plant breeding. Though *in-vitro* selection of somatic mutations is advantageous in plant breeding, few successful attempts have been made. Applications of somatic mutation in plant breeding could become valuable provided *in-vitro* selection and rapid plant screening methods are available. Cultivars of agronomically important crops, e.g. *Oryza sativa*, have been produced through the exploitation of protoclonal variation.

Ogura *et al.* (1989) reported the characterization and presence of protoclonal variation in *Oryza sativa* using the original regenerated plants from protoplasts. The common characteristics of these protoclones were: a slight increase in the number of panicles, a slight decrease in the number of spikelets per panicle, and no difference in grain yield. These results were encouraging for *Oryza sativa* breeding, using protoplasts and genetically manipulated plants. After confirming the stable inheritance of the favourable phenotypes (grain quality, plant height and plant vigour) as the protoclonal variation, the new *Oryza sativa* cultivar 'Hatsuyume' was developed in 1990, using for the first time a new strategy of 'protoplast breeding'. Following its introduction, commercial varieties in *Oryza sativa* developed from protoclones have been released by several companies, for instance 'Yumekaori' in 1993 (registration no. 3573), and 'Hareyaka' in 1995 (registration no. 4413).

Conclusion

Recent advances in protoplast culture and its applications in plant molecular biology have produced several opportunities for crop improvement. A regenerable

protoplast system would be suitable for the selection of useful variants, interspecific and intergeneric protoplast fusion and gene transformation. Improvements in molecular biology have made a contribution towards the introduction of the new functional genes, for example, herbicide resistance (Botterman and Leemans, 1988) and insect resistance (Hilder *et al.*, 1987), into regenerated plants. For transformation of new genes into protoplasts, several methods have been improved, that is, electroporation (Fromm *et al.*, 1986), treatment of polyethylene glycol (Lorz *et al.*, 1985) etc. The alternative transformation methods in plant tissues, that is, *Agrobacterium* system (Hernalsteens *et al.*, 1980) and gene gun system (Klein *et al.*, 1987) etc., have been developed in plant tissues. These systems are useful when protoplast culture has not been well developed on the target species. The systems of protoplast culture also have a wide range of adaptability for the development of new materials using cell fusion techniques. Based on increases in application of protoplast culture system in many species, somatic hybrids have been developed in several combinations of species, for example, between *Lycopersicon esculentum* and *Solanum acaule*, between *Oryza sativa* and *Echinochloa oryzicola*, etc. (Schweizer *et al.*, 1988; Terada *et al.*, 1987). Kunitake *et al.* (1995) reported that the protoclones of *Limonium perezii* had morphological traits such as leaf shape, flower stalks and calyces rather than the meristem-derived plants. Hence, genetic variation is more likely to be induced through protoplast culture than other culture systems.

The development of two varieties of *Oryza sativa* derived from protoclonal variation is a good example of the potential of this technology. In order to develop this technology as a major system of crop improvement, it is important to control undesirable phenotypic or genetic changes in the regenerated plants. This problem could be overcome by adequate culture techniques and suitable selection methods to minimize undesirable material, or to eliminate them by applying early selection pressure. The control of genetic changes during protoplast culture is an important factor in the induction of desirable mutants for crop breeding. Since DNA rearrangement during protoplast culture should be considered as the main cause resulting in protoclonal variation, molecular analysis of the mechanisms of genetic rearrangements during protoplast culture is expected to provide useful information regarding this technology in crop improvement. Advances in protoplast culture and molecular marker analysis will further enhance the efficiency of protoclonal variation in crop improvement.

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9. Chromosomal Basis of Somaclonal Variation in Plants

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Introduction

Plant regeneration from calli, cell suspensions and/or protoplasts has been extensively utilized in recent years both for the production of transgenic plants and for generating somaclonal variation (Jain, 1993a,b, 1997; Karp, 1995). This genetic variation is important in crop improvement, but also causes problems in maintenance of genetic fidelity during long-term *in-vitro* conservation of germplasm, in clonal propagation of germplasm and in the production of transgenic plants. The tissue culture-induced heritable variation, unlike the mutagen-induced variation, is often also associated with chromosome instability (Larkin and Scowcroft, 1981; Karp, 1991; Lee and Phillips, 1988; Peschke and Phillips, 1992; Singh, 1993). Although the causes of this chromosome instability are poorly understood, chromosome instability itself is believed to be one of the most common causes of tissue culture-induced variation (Phillips *et al.*, 1994). In cell cultures a complete range of chromosomal aberrations is known to occur; however, due to reduced morphogenic potential and reduced viability of aberrant cultured cells, the range and frequency of these aberrations is generally reduced in regenerated plants.

The literature on chromosomal variation originating in plant tissue cultures was earlier reviewed separately for cultured cells (Sunderland, 1973, 1977; Bayliss, 1980), and for regenerated plants (D'Amato, 1977). However, more recent reviews deal with chromosomal variation in cultured cells as well as regenerated plants, and present several hypotheses to explain the origin of these chromosomal variations (Lee and Phillips, 1988; Singh, 1993; Phillips *et al.*, 1994). In this chapter, chromosomal aberrations as observed in cell suspensions, in calli and in the regenerated plants are reviewed. Causes of these chromosomal aberrations and their relationship with the heritable phenotypic variation in the regenerated plants are also discussed.

Chromosomal Variation in Cultured Cells

Although variable chromosome numbers observed in fresh root tips, pith and hypocotyl tissues may account for part of the chromosomal aberrations in culture, generally the chromosomal aberrations are induced only during the culture. The possibility of numerical and structural changes in chromosomes originating in culture was initially discussed by Partanen (1963). In subsequent studies, considerable variation in the nature and extent of chromosome changes was observed in cultured cells. For instance, it has been shown in several studies that, among

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cultured cells, structural changes in chromosomes are more frequent than numerical changes. Sometimes the structural changes gave rise to dicentric chromosomes due to fusion events, as demonstrated in *Crepis capillaris* and *Apium graveolens* (Ashmore and Gould, 1981; Murata and Orton, 1983). Ring chromosomes, acrocentrics, telocentrics and dicentric chromosomes have also been observed in embryo-derived callus of barley (Singh, 1986; Fig. 1).

Analysis of structural changes of chromosomes suggested that the breakpoints are not random and that specific regions of chromosomes carrying heterochromatin blocks are often involved in chromosome rearrangements. For instance, a high proportion of these aberrations involve chromosomes with nucleolar organizing region (NOR), as shown in *Crepis capillaris* (Sacristan, 1971) and *Zea mays* (Lee and Phillips, 1987). Similarly, in maize, non-random rearrangements involving chromosomes 7 and 8 have been reported (Benzion *et al.*, 1986). Non-random chromosome rearrangements have also been reported in triticale (Brettell *et al.*,

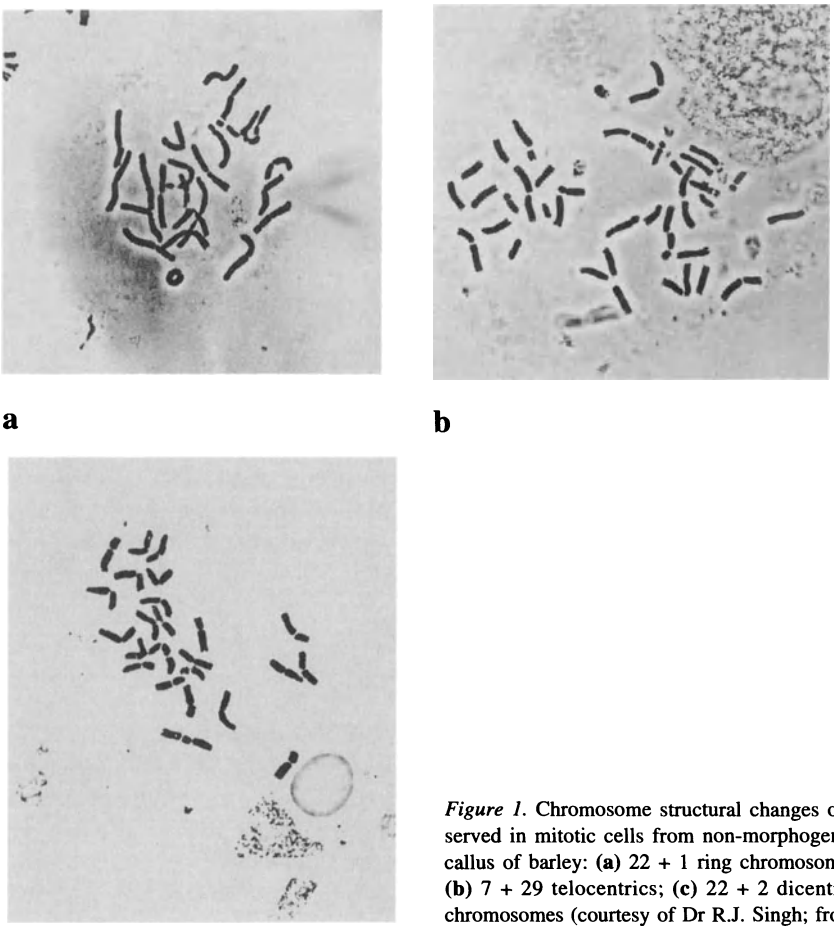


Figure 1. Chromosome structural changes observed in mitotic cells from non-morphogenic callus of barley: **(a)** 22 + 1 ring chromosome; **(b)** 7 + 29 telocentrics; **(c)** 22 + 2 dicentric chromosomes (courtesy of Dr R.J. Singh; from Singh, 1986)

1986), celery (Murata and Orton, 1983) and oats (Johnson *et al.*, 1987). This feature of non-random chromosome rearrangements, however, is not unique to tissue culture-induced chromosome rearrangements, since non-random distribution of breakpoints has also been shown in artificially induced interchanges in maize (Longley, 1961), *Vicia faba* (Rieger *et al.*, 1975) and tomato (Gill *et al.*, 1980).

A range of variation has also been reported for tissue culture-induced numerical changes in chromosomes. While alterations in chromosome number were reported in some studies (Murashige and Nakano, 1967; Heinz *et al.*, 1969; Kao *et al.*, 1970), no such variation was observed in several other studies (Kao *et al.*, 1970). For instance, in barley callus derived from embryo, Singh (1986) reported cells with different ploidy levels (Fig. 2). The causes of these numerical

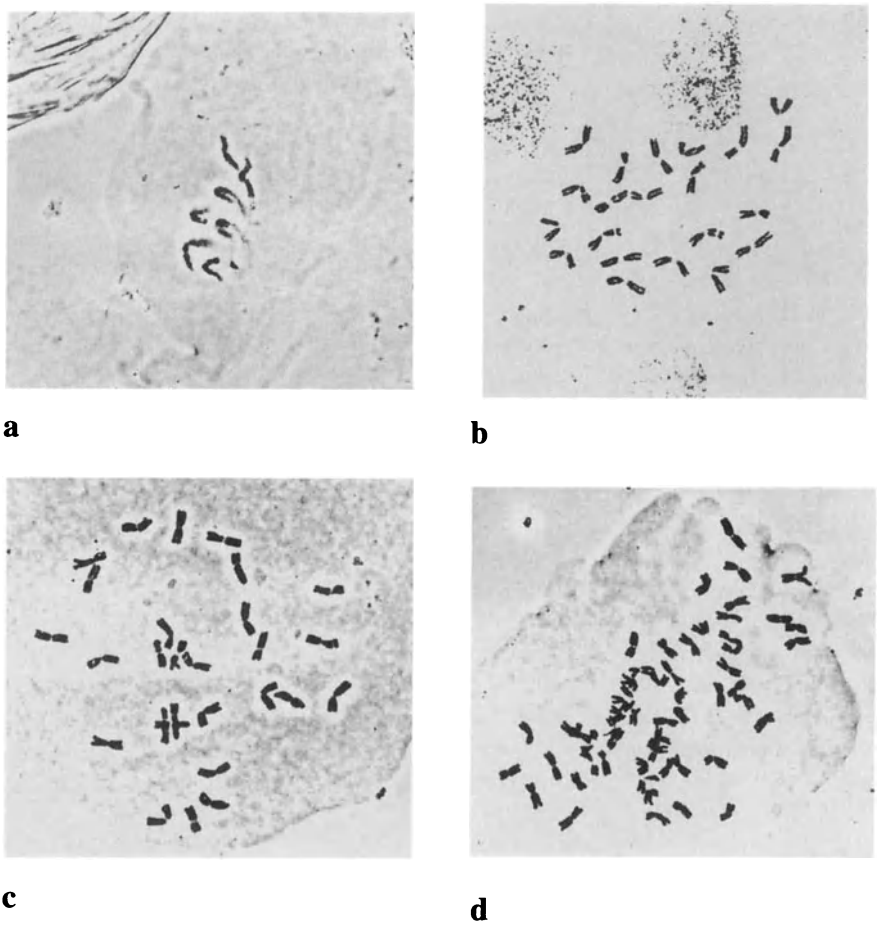


Figure 2. Chromosome numerical changes showing different ploidy levels observed in mitotic cells from morphogenic callus of barley: **(a)** haploid ($2n = x = 7$); **(b)** triploid ($2n = 3x = 21$); **(c)** tetraploid ($2n = 4x = 28$); **(d)** octoploid ($2n = 8x = 56$) (courtesy of Dr R.J. Singh; from Singh, 1986)

changes in chromosomes observed in tissue culture are not fully understood, although several hypotheses have been proposed (Lee and Phillips, 1988; Phillips *et al.*, 1994).

Growth of Telomere in Barley Callus Culture

A recent study in barley cv. Golden Promise demonstrated that the telomeres get elongated in callus cultures derived from immature embryos (Kilian *et al.*, 1995). The DNA was extracted from the callus and subjected to Southern blot (after *Mbo*I digestion) and dot–blot analyses using the probe $(CCCTAAA)_{25}$ to determine the telomere length and the number of telomere repeats, respectively. Both the terminal restriction fragment (TRF) length and the number of telomere repeats (TR) increased with the age of the callus. The roles of telomere binding protein in the elongation of the telomere in culture and its reduction during differentiation have been emphasized.

Factors Affecting Chromosomal Aberrations in Tissue Culture

A chromosomal aberration may originate either in the tissue used as explant, or in the subclone tissue derived from this explant, or even during the process of regeneration (D'Amato, 1977). Results of some of the experiments conducted to discover the stage at which chromosomal aberrations originate in culture are summarized in Table 1. Cells with cytological aberrations may replace the normal cell type, or may be eliminated, or may even maintain themselves in the culture. The extent of chromosomal variation also depends upon many other factors such as constitution of culture medium, the nature of the culture (organogenic vs. embryogenic), the species and genotype used, the tissue used as explant, the age of the culture, etc.

Constitution and Nature of Culture Medium

Chromosomal aberrations are believed to be induced in culture due to the effect of chemical substances in the medium, although the extent of the effect is modified by several other factors (for details see Singh, 1993). It has also been shown that more chromosomal abnormalities are induced in liquid medium (suspension cultures) than in the solid medium as demonstrated in carrot (Mitra *et al.*, 1960) and wheat (Karp *et al.*, 1987b).

Nature of Callus (Morphogenic vs. Non-morphogenic) and Genotype of Explant

The chromosome constitution of cultured tissue also depends on the nature of callus. Morphogenic callus generally exhibit a balanced chromosome number, while non-morphogenic callus exhibit high frequency of polyploidy and aneu-

Table 1. Chromosomal aberrations pre-existing in explants or induced during culture in different plant materials

Plant material	Explant	Reference
Pre-existing chromosomal aberrations in explants		
Allium cepa	Root tips	Partanen, 1963
Pisum sativum	Root tips	Torrey, 1959
Nicotiana tabacum	Pith tissue	Shimada and Tabata, 1967 Murashige and Nakano, 1967
Haplopappus gracilis	Hypocotyl	Singh & Harvey, 1975
Chromosomal aberrations induced during the culture		
Hordeum vulgare	Immature embryo	Singh, 1986
	Single cell clones	Cooper *et al.*, 1964
Nicotiana tabacum	Pith tissue	Shimada & Tabata, 1967
Haplopappus gracilis	Suspension culture	Singh and Harvey, 1975
Zea mays	Mesocotyl	Balzan, 1978
× *Triticosecale Wittmack*	Callus culture	Armstrong *et al.*, 1983
Solanum tuberosum	Protoplast culture	Sree Ramulu *et al.*, 1984
	Leaf segments	Pijnacker *et al.*, 1989
Pisum sativum	Immature embryos	Natali and Cavallini, 1987

ploidy. This has been observed in *Daucus carota*, *Hordeum vulgare*, *Haworthia setata*, *Nicotiana tabacum*, *Pisum sativum*, *Triticum aestivum* and *Zea mays* (reviewed in Singh, 1993). In wheat, it has also been shown that the cultures derived from aneuploid stocks carried more chromosomal aberrations than those derived from euploid wheat stocks (Asami *et al.*, 1975). Cell suspensions of diploid, tetraploid and hexaploid wheats have also been used for a comparison of chromosome instability at different ploidy levels (Winfield *et al.*, 1993). Similarly, in potato, cultures derived from monohaploids ($2n = x = 12$) carried more polyploids ($1x \rightarrow 2x \rightarrow 4x \rightarrow 8x$) than those derived from dihaploids/tetraploids (Sree Ramulu *et al.*, 1985). The genotype of the explant also influences the induction of chromosome instability in the culture and its regenerability (reviewed in Lee and Phillips, 1988; Singh, 1993). In a recent study using two inbred lines of maize also, it was shown that the genotype with higher level of knobs had relatively higher frequency of aberrant cells in culture (Fluminhan and Kameya, 1996).

The degree of chromosome instability in tissue culture also varies from one species to another. For instance, rye exhibits more chromosome instability than either barley or pearl-millet. This instability in rye is attributed to repetitive sequences located in the heterochromatin in rye genome. Further, the instability may also depend on the inbred/outbred nature of the genotype as shown in rye, since it is assumed that some of the genetic factors causing instability may be eliminated during inbreeding. However, in some inbreds also, instability

could be high (Puolimatka and Karp, 1993), suggesting that factors other than the breeding system may also be responsible for chromosome instability in tissue culture.

Age of callus

Several studies indicate that the age of callus affects the frequency of chromosomal aberrations. In general, the frequency of chromosomal aberrations increases with the age of the callus. However, in maize it has been shown that ageing did not influence the frequency of chromosomal aberrations (Table 2).

Variation in Chromosomes and in Genome Size in Tissue Culture-regenerated Plants

The plants regenerated from somatic cells, anthers or from protoplasts have been found to exhibit phenotypic variation, that can be attributed to epigenetic, genetic and chromosomal changes. A majority of regenerated plants, harbouring structural and numerical aberrations, also exhibit phenotypic variation. These alterations, particularly the numerical changes (aneuploidy and polyploidy), are more common among polyploids (e.g. sugarcane, wheat, oats, triticale, potato and tobacco) than in diploids, since the latter cannot easily tolerate aneuploidy (Tables 3 and 4). The only exceptions are *Pisum sativum* (nine of 20 regenerants were mixoploid), *Brassica oleracea* (of 71 regenerants six were diploid, 54 tetraploid and 11 octoploid) and *Secale cereale* (of 97 regenerants, 17 were mixoploids, three were diploid, one was haploid, one was aneuploid and six had chromosome breaks). The source of the explants may also contribute to polyploidy. For instance, more polyploid regenerants in diploid crops will be available if pith is used as an explant, since pith cells may carry diploid as well as polyploid cells (Murashige and Nakano, 1967). Among structural changes, translocation heterozygosity has recently been observed in somaclonal variants from immature embryo cultures in barley (Zhang *et al.*, 1994) and wheat (Franzone *et al.*, 1996). Variation in telomeric heterochromatin has also been reported in the seed progeny of 50 rye regenerants, using *in-situ* hybridization (Karp *et al.*, 1992).

Changes in chromosomes structure during culture or in the regenerants have also been examined at the DNA level as initially exemplified in the regenerated doubled haploids of *Nicotiana* spp. (De Paepe *et al.*, 1982; Dhillon *et al.*, 1983). These alterations in genome size generally involve the non-coding repetitive DNA, but rarely may involve the functional genes also (for references see Cavallini *et al.*, 1996). Recently, alterations in genome size in plants regenerated from tissue culture were reported in pea (Cecchini *et al.*, 1992; Bernardi *et al.*, 1995; Cavallini *et al.*, 1996). It was also shown that increase in DNA content was associated with increase in chromosome length at mitotic metaphase.

Table 2. The effect of the age of callus/cell suspension on the induction of chromosomal aberrations

Plant material	Age of callus	Percent cells with chromosomal aberrations			Reference
		Haploidy	Polyploidy	Aneuploidy	
Callus					
Nicotiana tabacum (2n = 48)	0 years (pith freshly excised)	–	68	2 (2n = 182, 184)	Murashige and Nekano (1967)
	1 year	–	(20%) (2n = 4x, 8x)	(53.3%) (2n = 54–304)	
	6 years	–	–	100% (2n = 108–174)	
Hordeum vulgare (2n = 14)	1 day	–	–		
	14 days		2.3 (4x)	~1%	Singh, 1986
	3–7 months	–	–	(36%)	Shimada, 1971
	2 years	–		(100%)	Shimada, 1971
Allium sativum (2n = 16)	92 days	0.75	14.18	31.34	
	157 days	1.51	34.85	17.43	Novak, 1974
	254 days	1.59	33.33	22.22	
	339 days	2.03	45.27	26.35	
Apium graveolens (2n = 22)	6 months	Chromosomal aberrations		(16%)*	Orton, 1985
	12 months	Chromosomal aberrations		(97.5%)*	
Zea mays (2n = 20)	4 months	Chromosomal aberrations		(3–4%)*	McCoy and Phillips, 1982
	8 months	Chromosomal aberrations		(5–6%)*	

Table 2. The effect of the age of callus/cell suspension on the induction of chromosomal aberrations (contd)

Plant material	Age of callus	Percent cells with chromosomal aberrations			Reference
		Haploidy	Polyploidy	Aneuploidy	
Cell suspension					
Triticum aestivum	6 months	–	–	65%	Winfield *et al.*, 1993
(2n = 42)	15 months	–	–	100%	
	31 months	–	–	60%	
T. dicoccum	4 months	–	–	~45%	Winfield *et al.*, 1993
(2n = 28)	12 months	–	–	~35%	
	31 months	–	–	~25%	
T. durum (2n = 28)	6 months	–	–	~10%	Winfield *et al.*, 1993
	15 months	–	–	~70%	
	32 months	–	–	~40%	
T. tauschii	6 months	–	4%	~40%	Winfield *et al.*, 1993
	14 months	–	6%	~20%	
T. monococcum	5 months	–	–	~5%	Winfield *et al.*, 1993
	12 months		25%	30%	
Haplopappus gracilis	7–258 days	–	3.6–36.5	3.5–10.0	Singh and Harvey, 1975

* Cells with haploidy, polyploidy and aneuploidy were not scored separately

Table 3. Structural changes in chromosomes observed in regenerated plants

Species	Number of regenerated plants	Analysis	Number of regenerated plants with chromosonal aberrations*			
			Translocation	Deficiency	Duplication	Other†
Diploids						
Zea mays L.	370	Meiotic	23	13	0	1
	267	Meiotic	45	59	0	1
	257	Meiotic	32	36	0	0
	142	Meiotic	7	0	0	0
	110	Miotic	0	0	0	0
Polyploids						
Triticosecale Wittmack	51	Mitotic	3	29	0	0
Wheat–rye hybrid	10	Mitotic	4	10	6	0
Avena sativa L.	799	Meiotic	48	180	0	2

(modified from Lee and Phillips, 1988)

*'Aberration' = number of plants containing that aberration.

†'Other' includes inversions, centric fusions, and more complex, unclassified rearrangements.

Table 4. Summary of numerical changes in chromosomes among regenerated plants .

Species	Frequency of regenerants (Percent with variable chromosome number)			Total	Reference
	Euploids	Mixoploids	Aneuploids		
Diploids					
Apium graveolens	0	0	2.0	4.3	Orton, 1985
Hordeum vulgare	0	0	0	Nil	Karp *et al.*, 1987a
Lolium multiflorum	0	0	0	1.9	Jackson *et al.*, 1986
Lolium multiflorum	0	0	0	Nil	Jackson and Dale, 1988
Lotus corniculatus	3.0	6.0	0	9.0	Damiani *et al.*, 1985
Pennisetum americanum	0	0	0	Nil	Swedlund and Vasil, 1985
Pisum sativum	0	45.0	0	45.0	Natali and Cavallini, 1987
Brassica oleracca	91.5	–	–	91.5	From Singh, 1993
Secale cereale	1.0	17.5	1.0	19.5	Lu *et al.*, 1984
Sorghum arundinaceum	0	0	0	Nil	Boyes and Vasil, 1984
Zea mays	0	0	1.8	1.8	Edallo *et al.*, 1981
Zea mays	0	2.4	1.6	4.0	McCoy and Phillips, 1982
Polyploids					
Avena sativa (6x)	0	–	7.76	7.76	McCoy *et al.*, 1982
× *Triticosecale* (6x)	0	–	38.9	38.9	Armstrong *et al.*, 1983
*Hypericum perforatum** (4x)	11.1(2x), 27.2(3x)	9.8	rare	48.1	Cellarova and Brunakova, 1996

(modifed from Singh, 1993)

*Only somaclones were cytologically analysed.

Chromosomal Aberrations in Haploid Callus and in Anther/Microspore-derived Haploid Plants

Chromosomal aberrations (both numerical and structural) are more frequent in calli and regenerants derived from anther/microspore than in the calli derived from somatic tissues or in their regenerants. This has been shown in many cases including *Crepis capillaris* (Sacristan, 1971). It has also been shown in a number of cases that the anther-derived plants, particularly when derived through a callus phase, have a sporophytic chromosome number due to chromosome doubling during regeneration. These chromosomal aberrations can also be utilized for chromosome engineering as discussed for the *Triticeae* (Hu, 1996). Some of the results of chromosome analysis of plants derived from anther culture are given in Table 5.

Chromosomal Aberrations in Callus and Regenerants Derived from Protoplasts

Cytogenetic basis of protoclonal variation has also been studied in several plant systems including potato, rice, *Brassica*, etc. The protoplasts could be derived from somatic tissue or gametophytic tissue for regeneration of plants exhibiting this protoclonal variation. Sometimes, they have been isolated from androgenic callus. Chromosome analysis has only rarely been conducted on callus cultures derived from protoplasts. More often, such an analysis has been conducted on plants regenerated from protoplasts.

In potato, several studies using different tetraploid cultivars (e.g. Maris Bard, Fortyfold, Bintze, Russet Burbank) and some dihaploids have been utilized for the study of the chromosomal basis of protoclonal variation. Aneuploidy in potato regenerants derived from protoplasts varied from a low (Karp *et al.*, 1982) to a high frequency (Sree Ramulu *et al.*, 1983, 1984). The frequency of aneuploidy also varied from a low of 20% in the normal-looking potato regenerants to a high of 82% in the morphological variants in a specific study (Sree Ramulu *et al.*, 1983, 1984). Mixoploidy (Sree Ramulu *et al.*, 1983, 1984) and structural changes (Gill *et al.*, 1985, 1986) were also reported. In another study on potato, while 100% of dihaploid regenerants exhibited variation in chromosome number, only 60% of the tetraploid regenerants exhibited such a variation, which was invariably associated with morphological changes (Sree Ramulu *et al.*, 1986). Similarly, in a study conducted in rice, among, 15 regenerants derived from protoplasts isolated from cell suspension of anther culture, only four were haploid and the remaining 11 were diploid (Toriyama *et al.*, 1986).

In the above cases, protoclonal variation is associated with variation in chromosome number. However, all morphological variation in protoclones cannot be explained on the basis of variation in chromosome number, since protoclonal variation was also available in plants with normal chromosome number ($2n = 48$). Such variation could be due to chromosomal structural changes, mutations,

Table 5. Chromosomal abnormalities in plants derived from anther culture

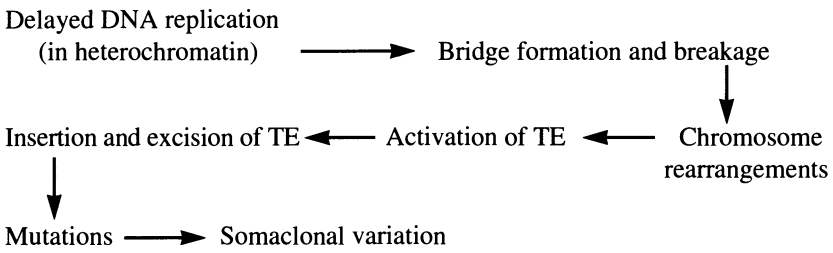
Species	Plants derived from	Percent plants with chromosome number				Reference
		Haploid	Diploid	Polyploid	Others	
Brassica napus (2n = 4x = 38)	Embryogenetic callus derived from anther	–	8.5	91.5	–	Sacristan, 1982
Solanum tuberosum (2n = 2x = 24)	Cell suspension from anthers	80.2	14.0	–	5.8	Uhrig, 1985
Solanum tuberosum (2n = 2x = 24)	Anther culture	–	–	–	–	Wenzel and Uhrig (1981)
	Cell suspension		2n = 33–151*			Pijnacker and Ferwerda, 1987
	Callus (2 yr old)		2n = 36–217*			
	Callus (morphogenic)		2n = 33–130*			
	Regenerants		2n = 34, 35*			
Pelargonium root (2n = 2x = 18)	Young callus	10.34	13.81	62.06	13.79	Bennici, 1974
	Old callus (anther derived)	19.04	71.42	4.76	4.76	
Nicotiana tabacum (2n = 4x = 48)	Anther culture		2n = 48 (mainly)*			Wersuhn and Sell, 1988
Secale cereale (2n = 2x = 14)	Anther culture	31.0	63.0	1.0	5.0	Wenzel *et al.*, 1977
Hordeum vulgare (n = 6x = 42)	Anther culture	3.0	97.0 (2x, 3x, 4x, etc)*			Mix *et al.*, 1978
Triticum aestivum (2n = 6x = 42)	Anther culture	85.8 (euploids)*			14.2	Hu, 1996
× *Triticosecale* (2n = 6x = 42)	Anther culture – callus	31.6	–	–	–	Pohler *et al.*, 1988
	Anther culture – embryoids	83.3	–	–	–	
Oryza sativa (2n = 2x = 24)	Protoplasts derived from (anther derived)	26.7				Toriyama *et al.*, 1986
	cell suspensions	73.3				
Oryza sativa (2n = 2x = 24)	Anther culture	49.1	35.7	5.1	10.2	Qiren *et al.*, 1985

(partly based on Singh, 1993)

mitotic crossing-over or chromosome segregation. Variation at the DNA level has also been detected in protoplast-derived grapevine regenerants through RAPD-PCR (Schneider *et al.*, 1996).

How Can Chromosome Breakage Cause Somaclonal Variation?

The above discussion has shown the association between chromosomal aberrations and somaclonal phenotypic variation in plants regenerated from plant tissue culture, but did not attempt to establish a causal relationship between the two. Chromosome breakage may be responsible for activation of transposable elements as initially documented in maize (Freeling, 1984). Once activated, transposable elements may cause a variety of genetic changes resulting in somaclonal variation. Activation of transposable elements *AC* and *Spm* (*En*) following tissue culture has actually been demonstrated (Peschke *et al.*, 1987). In view of this, Lee and Phillips (1988) suggested the following pathway for origin of somaclonal variation involving chromosome breakage:



Although reports of chromosomal aberrations in regenerated plants are available in several plant species (Tables 3 and 4), examples of definite association of such aberrations with somaclonal/protoclonal variations are few. However, any variation in regenerated plants would be useful to the plant breeder only when it is either due to a point mutation or due to stable change in chromosome structure or number. Therefore, any reports of polyploidy or aneuploidy cannot be the basis of useful genetic variation originating in tissue cultures.

Mechanism for the Origin of Chromosome Changes/Genetic Instability

Lee and Phillips (1988) summarized some of the studies involving elucidation of the possible mechanisms that may be involved in the origin of chromosome changes in plant tissue cultures. These mechanisms include the following:

Late-replicating Heterochromatin Hypothesis

According to this hypothesis, late replication of DNA, particularly in the heterochromatic segments of chromosomes, may be responsible for tissue culture-induced chromosome rearrangements. Such a mechanism was suggested by

Sacristan (1971) on the basis of her studies on *Crepis capillaris* callus cultures. She observed that 82% of the rearrangements involved the SAT-chromosomes, the breakpoints being located in the segments having delayed DNA synthesis. Similarly, the high frequency of telocentric chromosomes in regenerated oat plants was also attributed to delayed replication of pericentromeric heterochromatin (McCoy *et al.*, 1982). The association between late replication of heterochromatin and *in-vivo* chromosome breakage has also been demonstrated in maize (Pryor *et al.*, 1980) and *Vicia faba* (Rieger *et al.*, 1975). In maize, B-chromosome-induced chromosome breakage near the heterochromatin knob in A-chromosomes has also been attributed to late DNA replication. It was assumed that the late replication of DNA prevented separation of chromatids, thus leading to the formation of an anaphase bridge that caused breakage between the knob and the centromere (Rhoades and Dempsey, 1971, 1973). However, there are species which have little heterochromatin, but still showed a high degree of instability in culture, suggesting that other aspects of the genetic constitution may also play a role in chromosome instability in tissue culture (Karp *et al.*, 1992).

Nucleotide Pool Imbalance Hypothesis

According to this hypothesis, the plant cells in tissue culture may be prone to imbalances in dNTP pools, which partly explains the necessity of serial transfer of cultured tissues from old to fresh culture media. Such a hypothesis receives support from the observations in which human lymphocyte culture media deficient in thymidine and folic acid were shown to enhance the expression of fragile sites on chromosomes (Jacky *et al.*, 1983). Similar results were also obtained in Indian mole rat (Tewari *et al.*, 1987). Although plant cell cultures do not need exogenous thymidine and folic acid for growth, imbalances in dNTP pools, caused in some undefined manner under stress conditions, may be responsible for tissue culture-induced chromosome breakage.

Increased Mitotic Recombination Hypothesis

It has also been speculated that an increased frequency of mitotic recombination in plant tissue culture may cause culture-induced chromosomal aberrations (Larkin and Scowcroft, 1981; Lörz and Scowcroft, 1983). An increase in chiasmata frequency and a shift in the distribution of chiasmata have been shown in regenerated plants of rye, suggesting that increased genetic recombination may actually occur (Puolimatka and Karp, 1993). However, a direct correlation between enhanced mitotic recombination and origin of chromosomal aberrations in plant tissue cultures remains to be demonstrated.

DNA Methylation Hypothesis

DNA methylation patterns have been shown to be altered in plant tissue cultures (Brown *et al.*, 1991; Kaeppler and Phillips, 1993a,b). Both hypomethylation and

hypermethylation have been detected, utilizing methylation-sensitive restriction enzymes and several kinds of probes (both for single copy and repeat sequences). It was shown that the alterations in DNA methylation are not restricted to any specific kind of DNA sequences. This increased or decreased DNA methylation in cultures may lead to chromosome breakage, as inferred from a variety of observations including the following: (i) Heterochromatinization of chromosomes has been associated with increased methylation. (ii) Isolated nucleosomes containing histone protein H1 carry more methylcytosine than those without H1, as shown through the use of antibodies against methylcytosine (see review Phillips *et al.*, 1994). Because H1 is involved in chromosome condensation, an altered rate of DNA replication and an altered level of DNA methylation may indirectly cause anaphase bridges, chromosome breakage and rearrangement. (iii) Decreased methylation has been implicated in the non-disjunction of chromosomes, both in rye (Neves *et al.*, 1992) and *Neurospora* (Foss *et al.*, 1993). Thus, it is possible that chromosome breakage in plant tissue culture may be caused by breakdown of the control for the maintenance of the level of DNA methylation in the chromosomes.

Insertion or Excision of Transposable Elements

Earlier in this chapter evidence was presented, which suggested that chromosome breakage may lead to activation of transposable elements (TE) involving their insertion or excision-causing mutations. Insertion or excision of transposable elements within *in-vitro* systems, may also cause chromosome breakage leading to chromosomal aberrations. However, the role of this system causing chromosomal aberrations in cultured cells is not understood. Recently, in rice, a transposable element (*Tos17*) has been shown to be actively integrated within the genome during tissue culture, but the role of a naturally occurring TE in chromosome breakage has not been shown (Shimamoto, 1995).

Variability in Copy Number of Tandemly Repeated DNA Sequences in Plant Tissue Cultures

Plant tissue cultures also show variability in the copy number of tandemly repeated sequences (Landsmann and Uhrig, 1985; Brettell *et al.*, 1986). In recent years, variations in genome size involving non-coding repetitive DNA have been reported in regenerated plants of several plant species (Karp *et al.*, 1992; Kidwell and Osborn, 1993; Natali *et al.*, 1995; Cavallini *et al.*, 1996). Such variability is known to be a consequence of various cellular stresses inducing mitotic recombination and is also known to be the basis of several human diseases. In plant tissue culture, variability in copy number of tandemly repeated sequences is believed to be the cause of somaclonal/gametoclonal/protoclonal variation.

Thus, the cytological aberrations in tissue culture are associated with a variety of changes including chromosome breakage, activity of transposable elements, single base changes, alterations in copy number of repeated sequences and

changes in DNA methylation patterns (Lee and Phillips, 1988). It is possible that all these changes result from breakdown of a fine cellular control (Phillips *et al.*, 1994). The breakdown of normal controls is believed to be caused by the tissue culture environment. Tissue culture-induced changes in genomes can also be compared with responses of genomes to biotic/abiotic stresses, emphasized by McClintock (1984) in her Nobel lecture.

Conclusions

It is evident from the above discussion that numerical and structural changes in chromosomes, as well as alterations in genome size (nuclear DNA content), are induced both in tissue cultures and in the regenerants derived from cultures originating from somatic tissue, anthers/microspores and protoplasts. Telomeres have also been shown to elongate in tissue culture. Induction of these changes is influenced by several factors including nature of culture medium, nature of callus, age of callus, etc. It is suggested that chromosomal rearrangements may result from activation of transposable elements (TEs), DNA methylation or demethylation and duplication of repeated sequences. Such chromosomal rearrangement may be partly responsible for somaclonal variation. Thus, some progress in understanding the basis of tissue culture- induced heritable variation has been made in recent years. However, changes in chromosome number and structure may not be directly responsible for this variation.

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10. Somaclonal Variation and *In-vitro* Selection for Crop Improvement

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Introduction

Genetic variation is commonly found in tissue cultures. Plant tissues and cells cultured *in vitro* do mutate with a certain frequency. These mutations, however, are not always expressed and only a few of the alterations become evident as phenotypic and cytogenetical modifications in plants regenerated from the callus tissue (Bayliss, 1980; Lee and Phillips, 1988). Larkin and Scowcroft (1981) defined this type of variation as 'somaclonal variation'. Somaclonal variation has been accepted, and nowadays uniformity among the plantlets regenerated from callus tissue is considered to be the exception rather than the rule. However, as stated by Kreuger (1996), it is possible to regenerate true-type plantlets from *in-vitro* culture, for example, when plantlet formation is started from proembryogenic masses on auxin-containing medium. Most likely, somaclonal variation originates from the exposure of dedifferentiated tissues to culture cycles; the degree and expression of the variation depend on the genotype and the genetic base of the species (Karp, 1995).

Occurrence of variation, due to nuclear destabilization, seems to be dependent on tissue culture conditions. These conditions may be varied to minimize or maximize the extent of mutations. Such changes, either induced or spontaneous, provide additional variability to plant breeders, compensating for the progressively narrowing genetic base of many crop plants. However, somaclonal variation is being exploited as an alternative source of variation occurring under laboratory conditions for crop improvement as in *in-vitro* selection. The plant breeder does not have a precise tool in his hands and success can be increased by the right choices made during the selection process. What normally would take years and generations to accomplish under natural conditions could take place in a considerably shorter time; this is especially true for perennial crops. Selecting *in-vitro* minimizes environmental influence, and thus could supplement field selection, where populations derived from crosses are evaluated in field trials requiring an extensive amount of labour and space. *In-vitro* techniques may represent feasible integration of classical breeding techniques for crop improvement, specifically for biotic and abiotic stresses. Although, numerous examples are available on *in-vitro* selection, only a few somaclones have been successful as varieties. This reflects the major drawback of exploiting a source of variation difficult to use, whose outcome is not easily predictable and where a large part of variation originated is not stable or is highly undesirable. In 1981, Larkin and

S.M. Jain, D.S. Brar, B.S. Ahloowalia (eds.), Somaclonal Variation and Induced Mutations in Crop Improvement, pp. 169–201.

Scowcroft envisaged that somaclonal variation, together with *in-vitro* selection and early screening of regenerants, would provide a powerful option for plant improvement.

The Selection Process

Some mutations at the cellular level occur randomly and with a known frequency, between 5×10^{-8} and 2.5×10^{-7} (Thomas and Pratt, 1982; Singer and McDaniel, 1984). The types of mutations observed vary from point mutations (Evans and Sharp, 1983) to complete genome rearrangements, involving aneuploidy or polyploidy (Ahloowalia, 1976; Hartmann *et al.*, 1984a). It is extremely difficult to state whether these alterations are already present at the cellular level or if they occur during culture. It has been shown that callus tissue is composed of cells with different degrees of ploidy. The expression of the varied trait is not always evident, and plants regenerated from selected cell lines do not always express the desired trait. There are several causes for this phenomenon. For instance, the selected cell lines may have lost their totipotency and favour the regeneration of wild-type cell lines that survived in close contact to the mutated cells. These wild-type cells may take advantage of the favourable conditions and regenerate non-mutated plants. Alternatively, the cell lines may have simply habituated to the new growing conditions, tolerating momentarily the imposed stress conditions. Habituation-like epigenetic changes are not under genetic control and are commonly lost in the absence of selective pressure or after plant regeneration (Meins and Binns, 1978, 1982). A final possibility is that a back-mutation reverts the selected cell line to the wild-type (Melchers, 1971). These events may occur during the regeneration phase.

To exploit useful variation that occurs *in vitro*, the mutations must be transmitted to the progeny unchanged. Only in this case is it possible to speak of true somaclones. Therefore, the genetic nature of the somaclonal variation has to be tested. Plants regenerated from selected cells are defined as R_1 (R = regenerants) unless their nature as somaclone has been assessed. In this context S_1 (S = somaclone) will define all regenerants with some particular traits, distinct from the parent material. The successive generations are, therefore, named S_1, S_2 and S_3. When regenerants are screened for the desired trait, instead of their progenies, it implies that the selected trait is controlled either by a dominant gene or a heterozygous condition has reverted into a homozygous condition. On the contrary, recessive mutations can be detected only by screening the selfed progenies, and if they exhibit the selected trait, then it is heritable. According to Chaleff and Keil (1981), testing should even be carried out until the third generation, since some traits were observed to be lost in the S_2 generation.

Any *in-vitro* selection experiment should be preceded by the development of tests, providing an accurate measurement of the resistance or tolerance obtained during *in-vitro* selection. Wenzel and Foroughi-Wehr (1990) demonstrated the difficulty of evaluating the differential response of the selected regenerants or the

offspring of regenerants, compared with the parent genotype. A possible explanation for this phenomenon could be the inappropriate assays used to detect small quantitative alterations. These assays are basic for an accurate characterization of the genetic differences and quantitative alterations. Such tests should be reproducible, simple and offer maximal efficiency and minimal dependence on environmental factors. These assays should always be based on the results obtained from many genotypes and be tested with different assays. Differentiated tissues such as leaves, cuttings, roots and dedifferentiated tissues such as callus, cell suspensions or protoplasts should be included in the assays. These tests should determine whether the expression of the selected trait is comparable at the cell, callus or plant organ level.

A good general rule is to select at the lowest cellular organizational level to obtain solid mutants, implying that, for every crop, protocols for obtaining regenerable cell suspension cultures should be obtained. Since this is not true for all plant species, most researchers have done selection *in vitro* at the callus level, hoping that more variants would be obtained.

It is still controversial which screening method offers the best prospect for success. A number of papers report the isolation of disease-resistant somaclones derived either from the *in-vitro* selected or from the control batch (Brettell *et al.*, 1980; MacDonald and Ingram, 1985; Sacristan, 1982; Ling *et al.*, 1985; Shahin and Spivey, 1986; Toyoda *et al.*, 1989a; Binarova *et al.*, 1990). Whether selection should be applied before or after regeneration depends on the ability to efficiently screen the largest number of individuals with the minimal effort and maximal efficacy. *In-vitro* selection offers the possibility to screen thousands of putative variants in a Petri dish. The number of plantlets regenerated is limited, and during the successive evaluation phases, breeders may concentrate on a restricted number of individuals. In the situation in which the number of regenerated plants is considerably reduced by selective pressure, postponing the selection process until after regeneration may increase the chances of success. The choice between *in-vitro* selection and *in-vivo* screening should depend on the number of pros and cons connected to it. Exerting selective pressure may lead to the complete failure of regenerating plants with the desired trait; however, the second choice could imply that a major number of regenerants should be cultured, acclimatized and transferred to soil, with the consequent increase in costs, space, time and labour. The adoption of the optimal selective agent would be a favourable argument in favour of *in-vitro* selection, whereas when no specific agent is found, *in-vivo* screening of regenerants would be a better choice.

Even though *in-vitro* selection has been extensively investigated, the number of newly released cultivars is still very limited. For example, only four *Fusarium*-resistant somaclones have been registered up to now. The reason for this phenomenon is that the regenerants, though resistant, are frequently characterized by negative traits which are associated with the selected one. This is regardless of the fact that no true mutation is observed, but rather epigenetic changes. Shahin and Spivey (1986) regenerated toxin-resistant tomato plants which set seed. Their progenies showed a ratio of three *Fusarium*-resistant to one *Fusarium*-susceptible seedlings.

Types and Use of Selective Agents in the *In-vitro* Selection Process

Millions of potentially useful variants coexist in a cell population and could be theoretically screened *in vitro*. Therefore, a suitable selective agent is needed to efficiently isolate the cells which carry the desired trait. A selective agent should target a specific characteristic such as disease resistance. In addition, the ultimate goal of a good selection protocol is to achieve uniform selection, avoiding escapes, and reaching histogenic equilibrium, without losing the regeneration potential of the cells. So far, different selection protocols have been described, which try to mimic *in-vitro* the disease process or the stress-causing condition.

The pathogen itself may be chosen as a possible selective agent. However, in this case, infection problems can occur with the selected cells that have to be maintained for further growth and regeneration. Viruses have been applied to select virus-free regenerants (Murakishi and Carlson, 1982). In this research, the obtaining of uniform infection seems to be the major problem; infection frequencies appear to be around 90% (Harrison and Mayo, 1983). Sacristan (1979) developed a protocol in which callus of *Brassica napus* was inoculated with resting spores of *Plasmodiophora brassicae*, proposing this protocol as a selection method to isolate somaclones resistant to a pathogen. Under specific conditions, the pathogen may be successfully applied in *in-vitro* selection experiments (Prasad *et al.*, 1984), but overall the number of screening protocols involving the direct use of the pathogen is limited, and does not currently represent a valid alternative for using pathogen metabolites during *in-vitro* selection.

It has long been known that pathogens produce toxins during the infection process. A number of toxic metabolites have been studied and their role in plant pathogenesis revealed. However, not all pathogens produce a well-characterized toxin, and for some pathogens only culture filtrates have been applied in the selection process. The culture filtrate of a pathogen is a cocktail of all toxins and other metabolites produced by the fungus under artificial conditions. The heterogeneous composition of the filtrate may result in the selection of cells resistant to some toxic compounds which are not related to the disease. Consequently, mutant plants may not be resistant to the disease but to some metabolites of the pathogen as reported by Toyoda *et al.* (1984a,b). Furthermore, Vardi *et al.* (1986) raised reasonable doubts about the reliability of culture filtrates as selective agents in the *in-vitro* selection for *Citrus* variants resistant to *Phytophthora citrophthora*. Therefore, toxins that are well defined are preferable to culture filtrates as screening agents.

There is not a generally accepted definition of 'toxin', but in this context any metabolite exerting a toxic activity at very low concentrations on a plant cell or tissue falls into this definition. Toxins are basically divided into two broad classes according to Scheffer (1983). The first class includes host-specific toxins with a definite role in disease development. They are specifically toxic to susceptible genotypes of only one host species. Because of their specificity, such toxins are the most suitable selective agents. Regenerants, which are not affected by the toxin, are consequently also disease-resistant. However, the number of such

toxins is limited and they are mainly produced by some plant pathogens of the genera *Helminthosporium* and *Alternaria* (Scheffer, 1983). In addition, certain species of the bacteria, genus *Pseudomonas* are known to produce host-specific toxins. All the other toxins belong to the second class. These have a less well-characterized function, and need more careful evaluation in order to assess their role in the plant–pathogen interaction.

Toxins produced by the plant pathogens may have different activities. Understanding their role gives clues about the significance of these metabolites in the plant–pathogen interaction. Cercosporin produced by *Cercospora* spp. seems to act as a defensive compound produced by the pathogen rather than an offensive compound (Daub, 1986). In this case, *in-vitro* selection for resistance based on the use of this compound will not lead to disease-resistant regenerants. On the contrary, fusaric acid seems to be involved in the breakdown of the plant's defence mechanisms (Pegg, 1981). Therefore, fusaric acid-tolerant cell lines are more likely to also be *Fusarium*-resistant. Many toxins have been analysed for their potential to efficiently isolate resistant somaclonal variants. Among these toxins are deoxynivalenol (Wang and Miller, 1988), oxalic acid (Callahan and Rowe, 1991) and roridin E (Healey *et al.*, 1994).

For abiotic stresses, caused by excessive cold, heat and salt or presence of herbicides or heavy metals, the limiting factor can be used as a selective agent. Drought conditions *in vitro* have been achieved by adding either polyethylene glycol (PEG) or hydroxyproline to the medium (Heyser and Nabors, 1979). PEG is a non-metabolic, high molecular weight molecule which acts as an osmoticum, and therefore mimics the drought conditions. Hydroxyproline selects for proline accumulation; this amino acid is produced by the cells under general stress conditions (Sumaryati *et al.*, 1992).

Fundamental to attaining successful results during *in-vitro* selection is the manner in which the cells are challenged with the selective agents. Protocols for *in-vitro* selection are generally based on modification of a few basic selection schemes, such as direct and stepwise selection. Direct selection is done with a lethal concentration of the selective agent, while stepwise selection involves the progressive increase of the selective agent at sublethal concentrations, and is concluded when the lethal concentration is reached. Choosing between the one or the other strategy means having done a preliminary study, which should have given indications about reaction of the plant tissues when in contact with the selective agent. Concomitantly, as postulated by Daub (1986), more toxins need to be identified and characterized in order to detect more selective compounds with a role in pathogenesis and which may be used successfully in *in-vitro* selection.

Regeneration and Evaluation of Regenerants

After calli or cell lines are selected, the ultimate goal is to regenerate plants from these cells. Plant cells maintain their totipotent character for long periods, but under certain conditions their regenerative ability may be lost rapidly.

Modifications of the selection protocol may enhance the chances of regenerating plantlets from selected cells, i.e. when selective pressure is applied discontinuously instead of continuously. Chawla (1991) has stated that there are more advantages connected to the use of discontinuous selection than by using continuous selection. The alternation from selective medium to non-selective medium allows a partial recovery of the selected cells and increases the number of regenerated plants. Hartmann *et al.* (1984a) reported that selecting alfalfa calli in a rapid succession of selection steps reduced the chances of losing the regeneration ability of the calli, while increasing the chances of regenerating cytogenetically normal plants. The same observations were made for gladiolus cell suspension cultures challenged with fusaric acid (Remotti, 1996).

Once callus or cell lines are selected and plantlets are regenerated, the regenerants carrying the desired trait have to be retested and evaluated for stability. Initial testing can be done effectively at the callus level. After selection with toxins or culture filtrates, the calli may be challenged with the pathogen itself. Previous studies have shown that mycelial proliferation is inhibited by the callus of resistant varieties (Kroon *et al.*, 1991; Storti *et al.*, 1992). This method was used by Remotti (1996) to test the value of gladiolus calli selected for fusaric acid insensitivity. The selected cell lines, when inoculated with an isolate of *Fusarium oxysporum* f.sp. *gladioli*, retarded mycelial growth significantly.

Similarly, callus reinitiated from regenerants should display a differential behaviour when compared with the control callus, either surviving or growing better under adverse conditions. Regenerated shoots can be cultured in presence of the fungus (Miedaner *et al.*, 1987) or the number and extent of lesions produced by pathogens or toxic chemicals can be used as a measure of the increased resistance (Lind and Wenzel, 1984). To determine whether the resistance is stable, the progenies of somaclones or the vegetatively propagated somaclones have to be retested for resistance to the pathogen or pest. In the second case, the inheritance of the resistance may not be easily verified. In an extensive research, Hammerschlag (1988) selected peach somaclones *in vitro* for resistance to *Xanthomonas campestris* pv. *pruni.* These regenerants were micropropagated, rooted and tested under field and greenhouse conditions. In time, a small number of clones showed a decreasing expression of resistance, but the resistance was retained by part of the regenerants (Hammerschlag *et al.*, 1994). Hammerschlag thus succeeded in proving that stable changes may also be uncovered in vegetatively propagated crops.

In-vitro Selection for Disease Resistance

The first positive start signal for the application of *in-vitro* selection for disease resistance came from a report by Helgeson *et al.* (1972), who found a promising relationship between resistant tobacco varieties which produced only resistant calli, whereas susceptible varieties only generated susceptible calli. This report gave indications about the possible correlations between disease resistance and the reaction to the selective agent *in vitro.*

Table 1 shows examples of *in-vitro* selection for resistance to pathogens producing host-specific toxins. Fungi belonging to the genus *Helminthosporium* cause damage to a vast range of monocotyledonous species. Most of these fungal species produce well-characterized host-specific toxins and so in all reports of *in-vitro* selection, purified or partially purified toxin are used (Table 1). After the epidemic of 1970, caused by a new strain of *Helminthosporium maydis* which destroyed crops of those maize lines characterized by Texas cytoplasmic male-sterility, Gengenbach *et al.* (1977) succeeded in isolating mutants resistant to the HmT-toxins. This trait was maternally heritable and stable.

In several reports of *in-vitro* selection, a number of disease-resistant somaclones emerged among the control plants, indicating that the mutation related to the resistance occurred frequently. This is particularly useful for vegetatively propagated crops such as sugarcane, in which a vast array of morphological and biochemical mutants have been detected, along with novel genes for resistance to fungi and viruses (Tables 1 and 2).

Phytophthora infestans causes late blight in potato. Behnke (1979) was the first to report the selection and regeneration of resistant potato plants from culture filtrate-treated calli. In this case, a host-specific toxic compound is not produced by the pathogen. Calli were treated with the toxic metabolites of the fungus, and the regenerants proved to be resistant in the laboratory tests. Their resistance could not be fully assessed in field trials because of the occurrence of large epidemics of other diseases. Potato somaclones resistant to early blight, brown or grey leaf spot caused by *Alternaria* spp. have been regenerated from susceptible donor plants. Shepard *et al.* (1980) regenerated more than 10 000 potato plantlets from protoplasts, among which a number of disease-resistant variants were recovered. The trait was stable in the cloned regenerants. MacDonald and Ingram (1985) challenged secondary embryos of *Brassica napus* with a partially purified toxin of *Alternaria brassicicola.* They could also regenerate resistant plants from the control group, whereas Rodeva and Stancheva (1993) used culture filtrate to challenge callusing explants to detect resistant regenerants. One case of genetically transmitted resistance to *A. alternata* was induced in tobacco cells challenged with Al-toxins. Besides these successes, there is also one report by Witsenboer *et al.* (1988) in which tissue cultures, challenged with Al-toxin of *A. alternata* f.sp. *lycopersici*, failed to reflect the resistance at the whole plant level.

Carlson (1973) was the first to recover methionine sulphoximine-resistant tobacco from EMS-treated cells which were resistant to *Pseudomonas tabaci.* Bacteria belonging to the genus *Pseudomonas* produce well-characterized toxins, such as syringomycin and phaseolotoxin. These two metabolites have been used to select resistant plants (Gantotti *et al.*, 1985; Pauly *et al.*, 1987). However, transmission of resistance to the progeny was not reported. Thanutong *et al.* (1983) selected tobacco with partially purified toxins of *Pseudomonas syringae* pv. *tabaci. In-vitro*-obtained resistance appeared to be under the control of a few genes. Similarly, Toyoda *et al.* (1989b) selected tomato calli using a culture filtrate from *Pseudomonas solanacearum*; however, the type of inheritance of the recovered resistance in the regenerated plants was not specified.

Table 1. Examples of disease resistance to *Alternaria, Helminthosporium* and *Pseudomonas* obtained through *in-vitro* culture

Plant	Pathogen	Selective agent*	Resistance measurement on/by	Reference
Tobacco	*A. alternata* pv. *tabaci*	AL-toxin	Progenies	Thanutong *et al.*, 1983
Brassica	*A. brassicicola*	PPT, SR	Bioassay	MacDonald and Ingram, 1985
Carrot	*A. dauci*	SR	Bioassay	Dugdale *et al.*, 1993
Potato	*A. solani*	SR	Cloned regenerants	Matern *et al.*, 1978
	A. solani	SR	Cloned regenerants	Shepard *et al.*, 1980
Tomato	*A. solani*	CF	Progenies	Rodeva and Stancheva, 1993
Maize	*H. maydis*	HmT-toxin	Progenies	Gengenbach *et al.*, 1977; Conde *et al.*, 1979
	H. maydis	HmT-toxin, SR	Progenies	Brettel *et al.*, 1980
Rice	*H. oryzae*	HO-toxin, SR	Progenies	Ling *et al.*, 1985; Vidhyasekaran *et al.*, 1990
Sugarcane	*H. sacchari*	HS-toxin, SR	Cloned regenerants	Heinz *et al.*, 1977; Larkin and Scowcroft, 1983
Wheat	*H. sativum*	PPT	Bioassay	Chawla and Wenzel, 1987b, 1989
Barley	*H. sativum*	PPT	Progenies	Chawla and Wenzel, 1987b; Kole and Chawla, 1993
Oat	*H. victorae*	HV-toxin	Progenies	Rines and Luke, 1985
Celery	*P. chichorii*	SR	Bioassay	Wright and Lacy, 1988
Rice	*P. fuscovaginae*	Syringotoxin	Regenerants	Bouharmont, 1994
Tomato	*P. solanacearum*	CF, SR	Progenies	Toyoda *et al.*, 1989b
Tobacco	*P. solanacearum*	SR	Regenerants	Daub and Jenns, 1989
	P. syringae pv. *tabaci*	PPT	Progenies	Thanutong *et al.*, 1983
Bean	*P. syringae* pv. *phaseolicola*	Phaseolotoxin	Bioassay	Gantotti *et al.*, 1985
Wheat	*P. syringae* pv. *syringae*	Syringomycin	Regenerants	Pauly *et al.*, 1987
Peach	*P. syringae* pv. *syringae*	SR	Cloned regenerants	Hammerschlag and Ognjanov, 1990

*PPT, partially purified toxin; SR, screening of regenerants; CF, culture filtrate.

Table 2. Examples of disease-resistant somaclones selected *in vitro*

Plant	Pathogen	Selective agent*	Resistance measurement on/by	Reference
Pearl millet	*Claviceps fusiformis*	CF	Bioassay	Sharma and Chahal, 1990
Coffee	*Colletotrichum kahawae*	PPT	Regenerants	Nyange *et al.*, 1995
Barley	*Drechslera teres*	PPT	Progenies	Humhold *et al.*, 1990, 1992
Eggplant	*Mycoplasma*	Pathogen	Progenies	Mitra and Gupta, 1989
Poplar	*Melampsora larici*	CF	Bioassay	Antonetti and Pinon, 1993
Poplar	*M. allii-populina*			
Musa spp.	*Mycosphaerella fijiensis*	CF, PPT	Cloned regenerants	Okele and Schulz, 1997
Apple	*Phytophthora cactorum*	CF	Cloned regenerants	Rosati *et al.*, 1990
Strawberry	*P. cactorum*	CF	Cloned regenerants	Battistini and Rosati, 1991
Potato	*P. infestans*	PPT	Cloned regenerants	Wenzel *et al.*, 1984
Potato	*P. infestans*	CF	Cloned pants	Behnke, 1979, 1980a
Tomato	*P. infestans*	CF	Progenies	Mensurati *et al.*, 1991
Tobacco	*P. parasitica var. nicotianae*	CF	Regenerants	Daub and Jenns, 1989
Lemon	*P. tracheiphila*	PPT	Bioassay	Gentile *et al.*, 1992; Deng *et al.*, 1995
Brassica	*Phoma lingam*	CF, SR	Progenies	Sacristan, 1982
Strawberry	*Rhizoctonia fragariae*	Pectic enzymes	Cloned regenerants	Orlando *et al.*, 1997
Celery	*Septoria apiicola*	CF	Progenies	Evenor *et al.*, 1994
Soybeans	*S. glycines*	CF	Progenies	Song *et al.*, 1994
Tobacco	TMV	Virus	Progenies	Toyoda *et al.*, 1985, 1989a
Tomato	TMV	Virus	Progenies	Cassells *et al.*, 1986
Alfalfa	*Verticillium albo-atrum*	CF	Regenerants	Frame*et al.*, 1991
Hop	*V. albo-atrum*	CF	Regenerants	Connell and Heale, 1987
Eggplant	*V. dahliae*	CF	Progenies	Rotino *et al.*, 1990
Peach	*Xanthomonas campestris* pv. *pruni*	CF	Cloned regenerants	Hammerschlag, 1988

*CF, Culture filtrate; PPT, partially purified toxin; SR, screening of regenerants.

Most *Phytophthora*-resistant somaclones of vegetatively propagated crops such as apple, potato and strawberry were tested only on cloned regenerants (Table 2). Sexually transmitted resistance to *Phytophthora infestans* was recovered only in tomato by Mensurati *et al.* (1991). Cotyledons of a partially tolerant tomato variety were challenged with culture filtrate of *P. infestans.* All resistant plants regenerated were polyploid (F. Mensurati, personal communication). Wenzel *et al.* (1984) used partially purified toxin extracted from culture filtrate of *P. infestans* to regenerate resistant potato plantlets. The resistance of the cloned regenerants was constant *in vitro*, but in field trials the success of the *in-vitro* selection could not be determined, probably due to strong interference from other diseases. Similar problems have also hindered the final evaluation of the clones selected by Behnke (1979, 1980b).

Somaclones of various crops resistant to *Verticillium* wilts, brown spots and late blights caused by *Septoria* spp. have been reported with and without *in-vitro* selection (Table 2). The inheritance of the resistance was assessed in a number of cases (Rotino *et al.*, 1990; Evenor *et al.*, 1994; Song *et al.*, 1994).

Pathogens belonging to the genus *Fusarium* occur worldwide and the range of host plants attacked by this group of pathogens is vast. *In-vitro* selection for *Fusarium* wilt resistance has been the most studied (Table 3). Initially, investigations used the solanaceous plants tomato, potato and tobacco, as models, but nowadays *in-vitro* selection has been extended to a vast array of species. Up to now four *Fusarium*-resistant somaclones have been released as cultivars: a celery variant 'UC-T3' (Heath-Pagliuso *et al.*, 1989), the tomato selection 'DNAP-17' (Evans, 1989), a sweet potato somaclone 'Scarlet' (Moyer and Collins, 1983) and an alfalfa variety 'Sigma' (Varga and Badea, 1992). *Fusarium* spp. produce a large range of different metabolites with toxic effects on plants (Vesonder and Hesseltine, 1981). However, among them only fusaric acid (5-*n*-butyl-pyridine-2-carboxyl acid) and deoxynivalenol (DON) seem to play a significant role in disease development. Frequently, *Fusarium*-resistant genotypes are also fusaric acid-tolerant plants (Pesti *et al.*, 1987; Mégnégneau and Branchard, 1988; Arai and Takeuchi, 1993; Remotti and Löffler, 1996); the same holds true for *Fusarium* head blight resistance, in which resistant cultivars are DON-tolerant (Wang and Miller, 1988; Bruins *et al.*, 1993). Binarova *et al.* (1990) and Shahin and Spivey (1986) obtained somaclones of alfalfa and tomato resistant to *Fusarium* wilts from both *in-vitro* selected tissues and from the control material using either culture filtrate or fusaric acid.

Fusaric acid is a non host-specific toxin because it has been recovered from diseased tissues of different host plants, but its role in disease development has never been fully ascertained. Remotti (1996) investigated whether fusaric acid was involved in the disease development of *Fusarium* corm rot of gladiolus. Symptoms shown by *Fusarium*-infected gladioli are stunted growth related to partial or complete corm rot. The selection approach was preceded by a combined research approach. First the toxin present in the extract of diseased corms was detected and analysed, and GC–MS analysis confirmed the nature of the isolated compound. Fusaric acid was later used to discriminate *Fusarium*-resistant from *Fusarium*-susceptible genotypes in different assays based either on plant tissue or on callus

Table 3. Examples of *in-vitro* selection for *Fusarium* resistance

Plant	Pathogen and race	Selective agent*	Resistance measurement on/by	Reference
Wheat	*F. culmorum* and *F. graminearum*	Toxins	Progenies	Ahmed *et al.*, 1991
	F. culmorum and *F. graminearum*	DON	Regenerants	Bruins *et al.*, 1993
Lolium	*F. nivale*	DON	Progenies	Posselt and Altpeter, 1994
Celery	*F. oxysporum* f.sp. *apii*	SR	Progenies	Heath-Pagliuso *et al.*, 1989; Heath-Pagliuso and Rappaport, 1990
	F.o. f.sp. *apii* R2	SR	Progenies	Ireland and Lacy, 1987; Toth and Lacy, 1991
	F.o. f.sp. *apii* R2	SR	Regenerants	Wright and Lacy, 1988
Asparagus	*F.o.* f.sp. *asparagi, F. proliferatum*	SR	Cloned regenerants	Dan and Stephens, 1995
Sweet potato	*F. o.* f.sp. *batatas*	SR	Cloned regenerants	Moyer and Collins, 1983
Cucumber	*F. o.* f.sp. *cucumerinum*	CF	Bioassay	Malepszy and El-Kazzaz, 1990
Banana	*F. o.* f.sp. *cubense* R4	SR	Cloned regenerants	Hwang and Ko, 1989
	F. o. f.sp. *cubense* R1	FA	Cloned regenerants	Matasumoto *et al.*, 1995
Gladiolus	*F. o.* f.sp. *gladioli*	FA	Bioassay	Remotti and Löffler, 1996
Strawberry	*F. o.* f.sp. *fragariae*	SR	Cloned regenerants	Toyoda *et al.*, 1991
Tomato	*F. o.* f.sp. *lycopersici* R1	CF	Progenies	Scala *et al.*, 1984
	F. o. f.sp. *lycopersici* R2	SR	Progenies	Miller *et al.*, 1985; Evans, 1989
	F. o. f.sp. *lycopersici* R2, R3	FA, SR	Progenies	Shahin and Spivey, 1986
	F. o. f.sp. *radicis-lycopersici*	SR	Progenies	Rodeva and Stamova, 1993
Alfalfa	*F. o.* f.sp. *medicaginis*	CF	Regenerants	Hartmann *et al.*, 1984a,b
	F. o. f.sp. *medicaginis*	CF	Regenerants	Arcioni *et al.*, 1987
	F. oxysporum, F. avenacearum and *F. solani*	CF, SR	Regenerants	Binarova *et al.*, 1990
	F. o. f.sp. *medicaginis*	SR	Progenies	Varga and Bedea, 1992
Tobacco	*F. o.* f.sp. *nicotianae*	CF	Regenerants	Selvapandian *et al.*, 1988
Potato	*F. o.* f.sp. *solani*	CF	Cloned regenerants	Behnke, 1980b
Barley	*Fusarium* spp.	FA	Bioassay	Chawla and Wenzel, 1987a; Chawla, 1991
Red clover	*F. roseum*	CF	Regenerants	Constabel, 1989
Soybean	*F. Solani*	CF	Progenies	Jin *et al.*, 1996

*DON, dedeoxynivalenol; SR, screening of regenerants; CF, culture filtrate; FA, fusaric acid.

tissue. Finally, the pure toxin was used as a selective agent to isolate toxin-insensitive cell lines. Selected callus lines were inoculated with the pathogen and exhibited retarded mycelial growth, compared with control tissue. These results indicate that the fungus needs the toxin to condition the host tissues and prepare them for colonization. A definite answer about the involvement of fusaric acid in corm rot of gladiolus will be obtained when the selected plants are tested for *Fusarium* resistance in 1997 and 1998. According to Wenzel and Foroughi-Wehr (1990), it is most likely that the progenies of selected somaclones may not differ significantly from the starting material. To verify this, accurate resistance tests have been developed by Th.P. Staathof at CPRO-DLO, The Netherlands (personal communication). These tests should enable detection of slight increases of resistance among the regenerated plants.

In Tables 2 and 4, more examples of *in vitro*-selected variants or disease-resistant somaclones selected after regeneration are shown. For most of the host–pathogen interactions listed in the tables, a defined fungal metabolite, which could be involved in disease development, has not been found. Therefore, the reports of resistant somaclones recovered after regeneration out number the reports of somaclones selected directly *in vitro*.

There are a few reports describing innovative methods of selecting for disease resistance *in vitro*. Ben-Gweirif and Novacky (1987) succeeded in assessing hypersensitive reactions from cell suspension cultures of cotton challenged with a suspension of *Xanthomonas campestris* pv. *malvacearum*, measuring the electrolyte leakage of both resistant and susceptible controls. One additional protocol is based on the plating of cells or calli on a double layer of media. The first, lower layer is precolonized by the pathogen, autoclaved and covered with a second layer of callus growing medium. By this protocol, Ahmed *et al.* (1991) were able to select head-blight-resistant wheat plants. The toxin was supposedly thermostable and diffused through the upper layer. This protocol represents a valid alternative to the use of culture filtrates. However, if the selective agent is not thermostable this procedure is not effective. In that case, a technique proposed by Tomaso-Peterson and Krans (1990) may be used whereby the pathogen is physically separated from the plant tissues by a polycarbonate nucleopore membrane, through which the toxic metabolites may diffuse freely.

In-vitro Selection for Herbicide Resistance

The use of herbicides has become a common practice for weed control. A strategy is to develop herbicide-resistant cultivars at the same time as the development of the new herbicide. Of the various approaches, *in-vitro* selection for herbicide resistance could be used to obtain such resistant plants (Table 5). Natural variation in absence of selective pressure *in vitro* has so far been insufficient to generate a herbicide-tolerant regenerant. The active ingredients of the herbicides added to the tissue culture medium act concomitantly as selective and mutagenic agents. Mutagenic treatments have been shown to enhance the occurrence of resistant mutants (Sebastian and

Table 4. Examples of disease-resistant somaclones identified after plant regeneration

Plant	Pathogen	Resistance measurement on	Reference
Lettuce	*Bremia lactucae*	Progenies	Brown *et al.*, 1986
Celery	*Cercospora apii*	Regenerants	Wright and Lacy, 1988
Stylosanthes spp.	*Colletotrichum gloeosporoides*	Progenies	Godwin *et al.*, 1990
Kentucky bluegrass	*Erysiphe graminis*	Cloned regenerants	Msikita and Wilkinson, 1994
Poplar	*Melampsora medusae*	Regenerants	Prakash and Thielges, 1989
Potato	*Phytophthora infestans*	Cloned regenerants	Shepard *et al.*, 1980; Meulemans and Fouarge, 1986; Meulemans *et al.*, 1986; Cassells *et al.*, 1991
Sugarcane	*Puccinia melanocephala*	Cloned regenerants	Sreenivasan *et al.*, 1987
	P. melanocephala	Regenerants	Liu *et al.*, 1983
Wheat	*P. recondita*	Progenies	Oberthur *et al.*, 1993
Barley	*Rhynchosporium secalis*	Progenies	Pickering, 1989
Celery	*Septoria apiicola*	Regenerants	Wright and Lacy, 1988
Poplar	*S. musiva*	Cloned regenerants	Ostry and Skilling, 1988
Jerusalem artichoke	*Scerotinia sclerotiorum*	Cloned regenerants	Cassells and Walsh, 1995
Pearl millet	*Sclerospora graminicola*	Regenerants	Nagarathna *et al.*, 1993
Sugarcane	*S. sacchari*	Regenerants	Krishnamurthi, 1974
Potato	*Strepthomyces scabies*	Cloned regenerants	Jellis *et al.*, 1984; Evans and Sharp, 1986
Sugarcane	*Ustilago scitaminea*	Regenerants	Liu and Chen, 1978; Liu, 1981
Alfalfa	*Verticillium albo-atrum*	Cloned plants	Latunde-Dada and Lucas, 1983
Potato	*V. dahliae*	Cloned regenerants	Sebastiani *et al.*, 1994
Sugarcane	Fiji virus	Cloned regenerants	Krishnamurthi and Tlaskal, 1974; Heinz *et al.*, 1977
Potato	PVY, PLRV	Cloned regenerants	Jellis *et al.*, 1984
		Regenerants	Thomson *et al.*, 1986
	PVY, PVX	Cloned regenerants	Cassells *et al.*, 1986
Tomato	TMV	Progenies	Barden *et al.*, 1986; Smith and Murakishi, 1987
Lettuce	LMV	Progenies	Brown *et al.*, 1986

Table 4. Examples of disease-resistant somaclones identified after plant regeneration (contd)

Plant	Pathogen	Resistance measurement on	Reference
Tomato	*Clavibacter michiganense*	Regenerants	Zagorska *et al.*, 1993
Apple	*Erwinia amylovora*	Cloned regenerants	Donovan *et al.*, 1994
Peach	*Xanthomonas campestris* pv. *pruni*	Cloned regenerants	Hammerschlag, 1990; Hammerschlag and Ognjanov, 1990; Ritchie *et al.*, 1993
Geranium	*X. c.* pv. *pelargonii*	Regenerants	Dunbar and Stephens, 1989
Rice	*X. oryzae*	Progenies	Sun *et al.*, 1986

Chaleff, 1987; Saxena and King, 1988). This variation occurs at frequencies between 10^{-7} and 10^{-6}, (Thomas and Pratt, 1982; Harms and DiMaio, 1991) and different mutations may occur independently (Chaleff and Ray, 1984). The resistance mechanisms may be based on the specific mode of action of the herbicide. Somaclones that are resistant may have an altered transport ability, modified target enzyme or a detoxification mechanism. When the action of the herbicide is expressed at the cellular level, these mechanisms also hold true. Herbicides interfering with the photosynthetic electron transport system cannot be used for *in-vitro* selection unless the callus exhibits photosynthetic activity. Herbicides such as chlorsulphuron, whose site of action are specific enzymes such as acetolactate synthase, are the most successful, since they are effective at very low dosages.

Selection of herbicide-tolerant cell lines is easily accomplished, but most have not regenerated plants and relatively few have passed the resistance trait to their progeny (Table 5). Only when the progenies inherit the genes can the inheritance be assessed. From the literature resistance acquired *in vitro* is normally controlled by a single dominant gene. However, non-Mendelian patterns of inheritance have been reported (Singer and McDaniel, 1984), as have point mutations in the chloroplast DNA (Sato *et al.*, 1988). In other cases the selected tolerance can be characterized biochemically by identifying the altered forms of enzymes responsible for the tolerance. This has been the case of glyphosate tolerance in maize, in which a variation in the EPSP and DAHP was detected (Racchi *et al.*, 1995).

Different herbicides sharing a common site of action may be neutralized, the altered activity of certain enzymes conferring cross-resistance. Cross-resistance to sulphonylurea and imidazolinones in corn was conferred by decreased sensitivity of acetohydroxy acid synthase (AHAS) (Anderson and Georgeson, 1989) whereas in tobacco resistance to hydroxyurea and picloram were conferred by the *HuR* gene (Chaleff and Keil, 1981). Parker *et al.* (1990) selected corn lines resistant to two herbicides which inhibit acetylcoenzyme A carboxylase (ACCase). However, these findings do not exclude different mutations occurring in a somaclone or among somaclones of the same population (Chaleff, 1980).

Occurrence of susceptible plants after selection and regeneration is reported by Chaleff and Parsons (1978). This may be due to mutated cells protecting adjacent wild-type cells with diffusible substances or the presence of cells in clumps not directly in contact with the herbicide. Survival of these cells may also lead to chimaeras or partial resistance. For some herbicides, such as paraquat, some relatively easy, reliable and non-destructive assays are available at the early plant stage. While, for resistance to other herbicides, such as amitrol or glyphosate, the testing should be done at the whole plant level only (Hughes, 1983).

In-vitro Selection for Sodium Chloride and Salt Tolerance

Mineral stresses of plants, such as excess salts in soil and in water, represent a major source of crop yield loss. A large portion of arable lands is affected by high salt levels, particularly NaCl. Salt-tolerant plants exposed to a high saline environment

Table 5. Examples of *in-vitro* selection of herbicide-resistant somaclones

Species	Herbicide	Inheritance of resistance	Reference
Arabidopsis thaliana	Chlorsulfuron	Monogenic dominant	Haughn and Somerville, 1986
Beta vulgaris	Chlorsulfuron	Monogenic dominant	Saunders *et al.*, 1992
Brassica napus	Chlorsulfuron	Semi-dominant	Swanson *et al.*, 1988
Glycine max	Atrazine	Not single dominant	Wrather and Freytag, 1991
Linum usitatissium	Chlorsulfuron	Monogenic dominant	Jordan and McHughen, 1987
Lotus corniculatus	Chlorsulfuron	Monogenic dominant	Pofelis et al., 1992; Grant and McDougall, 1994*
Lycopersicon esculentum	Paraquat	Dominant mutations	Thomas and Pratt, 1982
Nicotiana tabacum	Picloram	Monogenic dominant	Chaleff and Parsons, 1978; Chaleff, 1980
N. tabacum	Hydroxyurea, picloram	Monogenic dominant	Chaleff and Keil, 1981
N. tabacum	Chlorsulfuron, sulfometuron methyl	Monogenic dominant	Chaleff and Ray, 1984
N. tabacum	Amitrole	Not Mendelian	Singer and McDaniel, 1984
N. tabacum	Atrazine	Chloroplast point-mutation	Sato *et al.*, 1988
N. tabacum	Paraquat	S_1 resistant	Furosawa, 1988
N. tabacum	Chlorsulfuron	Monogenic dominant	Caretto *et al.*, 1993
Oryza sativa	Bensulfuron methyl	Recessive mutations	Terakawa and Wakasa, 1992
Solanum tuberosum	MCPA, OMNIDEL	Cloned regenerants	Wersuhn *et al.*, 1987
Zea mays	Sethoxydim and haloxyfop	Semi-dominant	Parker *et al.*, 1990
Z. mays	Imidazolinone	Monogenic dominant	Newhouse *et al.*, 1991
Z. mays	Imidazolinone, Chlorsulfuron	Monogenic semi-dominant	Anderson and Georgeson, 1989
Z. mays	Glyphosate	S_2 resistant	Racchi *et al.*, 1995

*Somaclone released as cultivar.

suffer retarded development, low fertility and premature death. The application of tissue culture conditions provides a valuable method to increase salt tolerance in crop plants. Selection on media enriched with NaCl, KCl, Na_2SO_4, or other salts has isolated somaclones expressing stable acquired salt tolerance (Table 6). *In-vitro* selection for salt tolerance commonly achieves only a temporary adaptation; cells are able to compartment the excess salts into the vacuoles, and survive by adjusting the osmotic pressure in hostile environments (Flowers *et al.*, 1977), but this adaptation causes reduction of cell division and expansion (Bressan *et al.*, 1985). The limited understanding of both the mechanistic determinant of tolerance and the interrelationship between salt tolerance mechanisms and plant growth has hindered progress towards obtaining salt-tolerant crops. The relationship between salt tolerance and reduced cell expansion is also unknown. Among the inconveniences of adaptation to high salt conditions, it is possible to mention how some ecotypes or species have adapted so well to high salt conditions to become halophytic; these plants are now unable to complete their life cycle in the absence of salt.

Bressan and co-workers (1985) found that a critical concentration of NaCl would induce a reversible habituation instead of stable mutations. According to their findings, tobacco cells cultured in the presence of 10 g/L NaCl exhibited wild-type characteristics when the selection pressure was removed. Permanent salt tolerance was obtained with 25 g/L NaCl acting as selective and mutagenic agent. Increased NaCl treatments cause specific modifications of the plant morphology, including unbalanced polyploidization, sterility, longer reproduction phase and dwarfism coupled with heritable NaCl tolerance (Bressan *et al.*, 1985; McCoy, 1987). These chromosome alterations cause the death of the individuals. Despite these problems associated with the *in-vitro* selection for NaCl tolerance, a large number of salt-tolerant somaclones have been recovered from *in-vitro* selection (Table 6).

In some NaCl-tolerant regenerants, accumulation of an altered 24 kDa protein, called osmotin-I, was observed (Bressan *et al.*, 1987). Other reports observed an altered isozyme pattern or the presence of high levels of proline, an amino acid supposed to mediate the osmotic regulation of the cytoplasm (Stewart and Lee, 1974). Other salt-protective resistance mechanisms involved in *in-vitro*-selected salt-tolerant plants include the activation of chlorine-excluder genes (Abel, 1969), and the occurrence of osmoprotectants such as glycine-betaine (Storey and Wyn-Jones, 1975). A beneficial effect of challenging developing somatic embryos on medium supplemented with NaCl was observed in tobacco by Bressan *et al.* (1987), and in *Brassica juncea* when using high morphogenic cotyledons; in this case the progenies retained the salt tolerance (Jain *et al.*, 1990). The question of the agronomic value of selected genotypes is still open, and the proof is still lacking on the price paid by the plant for being more stress resistant.

Selection for Tolerance to Metals

Excess metal accumulation in agricultural soils causes problems in cultivated crops. Some metals are naturally predominant in acid soils, such as aluminium or

Table 6. Examples of *in-vitro* selection of sodium chloride- and salt-tolerant somaclones

Species	Selective treatment	Heritability of the selected triat	Reference
Beta vulgaris	7.6 g/L salts	S_1	Freytag *et al.*, 1990
Brassica juncea	5–10 g/L NaCl	S_1	Jain *et al.*, 1990
B. juncea	10–20 g/L NaCl	S_1	Kirti *et al.*, 1991
Citrus sinensis	2.92 g/L NaCl	S_0	Ben-Hayyim and Goffer, 1989
Coleus blumei	5.25 g/L NaCl	S_1	Ibrahim *et al.*, 1992
Colocasia esculenta	Various salts	S_0	Nyman *et al.*, 1983
Hordeum vulgare	10 g/L NaCl	S_5	Nabors and Dykes, 1985
Linum usitatissimum	2.5–3% salts	S_1	McHughen and Swartz, 1984
L. usitatissimum	2.5–3% salts	S_2	McHughen, 1987; Rowland *et al.*, 1988
Medicago sativa	10 g/L NaCl	Dominant trait in S_1	Winicov, 1991
Nicotiana tabacum	25 g/L NaCl	Dominant trait	Bressan *et al.*, 1987
N. tabacum	8.8–33.4 g/L NaCl	S_2	Nabors *et al.*, 1980
N. tabacum	11.7 g/L NaCl	Dominant trait in S_2BC_2	Sumaryati *et al.*, 1992
Oryza sativa	10 g/L NaCl	S_1	Narayanan and Sree Rangasamy, 1989
O. sativa	10–20 g/L NaCl	S_4	Vajrabhaya *et al.*, 1989
O. sativa	10 g/L NaCl	S_1	Winicov, 1996
Poncirus trifoliata	5–10 g/L NaCl	S_0	Beloualy and Bouharmont, 1992
Sorgum bicolor	5 g/L NaCl	S_1	Bhaskaran *et al.*, 1986

manganese. Acidification, caused by acid-forming fertilizers, solubilizes Al and Mg. Heavy metal contamination with Cd, Cu, Pb, Zn, and other metals, is mainly caused by human activity, and the presence of industrial waste; this creates the need to select for metal-tolerant crops. Crop plants tolerant to aluminium and manganese occur naturally (Foy, 1983). However, limited information is available on tolerance to heavy metals. *In-vitro* techniques offer possibilities to understand this phenomenon. The initial report was provided by Meredith (1978), who isolated tomato cell lines with enhanced resistance to aluminium. Subsequently, Al-resistant plants have been regenerated from various tissue cultures, challenged with Al salts *in vitro* (Table 7). The increased tolerance is frequently transmitted to the progenies. Ojima and Ohira (1982) were the first to regenerate Al-tolerant carrot plants selected with $AlCl_3$ *in vitro*. The carrot cells excreted citric acid, that acted as a chelating agent to reduce the concentration of ions in the root area. The tolerance mechanism enabled the plant to survive under adverse conditions, since the chelated metals lost their toxicity. However, when metals in excess are taken up by the roots and confined to the intercellular spaces, this compartmentation represents a potential threat to plant-consuming animals.

Plants tolerant to other metals have been studied. Cells of *Agrostis stolonifera* tolerant of Zn and Cu were observed to take up more metals than the non-tolerant cells. This tolerance was expressed either at the plant or at callus level (Wu and Antonovics, 1978). Not all species expressed the tolerance at the callus level (Christianson, 1979). Few studies have tried to isolate somaclones with tolerance to other heavy metals. Table 7 shows two examples of how cells may tolerate toxic levels of cadmium, but no plants have been regenerated from them.

In-vitro Selection for Environmental and Insect Stress

Limitations due to environmental stresses are caused mainly by water scarcity and temperature extremes. Several *in-vitro* stress-tolerant selections have been obtained (Table 7). Kishor and Reddy (1985) described a procedure based on the use of PEG, which provides osmotic stress and thus selects dehydration-tolerant cells. Plants of rice and wheat with increased tolerance to osmotic stress have been regenerated (Hsissou and Bouharmont, 1994; Adkins *et al.*, 1995). Inheritance of drought tolerance generated *in vitro* has not yet been investigated. A somaclone regenerated from abscisic acid (ABA)- treated calli of wheat was registered as a highly stress-resistant variety (Sears *et al.*, 1992). This selection is insensitive to endogenous levels of abscissic acid either at the seedling or adult stages. Under heat and drought stress line KS89WGRC9 presents unproved traits such as lower stomatal resistance and grain filling rate, along with other characteristics. Heat-tolerant cotton plants were selected by applying high temperatures (up to 45°C) to callus cultures (Trolinder and Shang, 1991). Plants were regenerated from surviving cells; callus could be induced from excised tissue at 38°C. Cytological abnormalities reduced fertility considerably and inheritance could not be tested.

Table 7. Examples of *in-vitro* selection for resistance to environmental stresses and insect resistance

Species	Types of tolerance achieved through *in-vitro* selection	Tolerance expressed in	Reference
Sorghum bicolor	Acid soil tolerance	S_7	Waskom *et al.*, 1990; Miller *et al.*, 1992; Duncan *et al.*, 1991b, 1995
Daucus carota	Al tolerance	S_1	Ojima and Ohira, 1982
Nicotiana plumbaginifolia	Al tolerance	S_1	Conner and Meredith, 1985a,b
Oryza sativa	Al tolerance	S_1	Okawara *et al.*, 1986; Bouharmount, 1994
O. sativa	Al tolerance	S_0	Nunez *et al.*, 1985
Solanum tuberosum	Al tolerance	S_0	Wersuhn *et al.*, 1988, 1994a,b
Datura innoxia	Cd tolerance	callus	Jackson *et al.*, 1984
Nicotiana tabacum	Cd tolerance	callus	Huang and Goldsbourgh, 1988
Oryza sativa	Cd tolerance	S_0	Li and Chen, 1987
Oryza sativa	Water stress	S_1	Adkins *et al.*, 1995; Kishor and Reddy, 1986
Triticum durum	Water stress	S_0	Hsissou and Bouharmont, 1994
Triticum aestivum	Water stress, heat tolerance	S_4*	Lu *et al.*, 1989; Sears *et al.*, 1992
Gossypium hirsutum	High T° tolerane (38°C)	S_0	Trolinder and Shang, 1991
Cucumis melo	Low T° germination (14°C)	S_3	Ezura *et al.*, 1995
Linum usitatissimum	Low T° germination (5–8°C)	*	O'Connor *et al.*, 1991
Medicago staiva	Cold hardiness (–16°C)	S_1	Nowak *et al.*, 1992
Oryza sativa	Cold hardiness (5–10°C)	S_1	Bouharmont, 1994
Trifolium pratense	Cold hardiness (–10°C)	S_2	Nowak *et al.*, 1992
T. aestivum	Frost tolerance (–8°C)	S_0	Dörffling *et al.*, 1993
T. aestivum	Frost tolerant (–12°C)	S_1	Kendall *et al.*, 1990
T. aestivum	Front tolerant (–13°C)	S_4	Galiba and Sutka, 1989
Zea mays	Cold tolerance (4°C)	S_0	Duncan and Widholm, 1987
Beta vulgaris	UV-B tolerance	S_0	Levall and Bornman, 1993
Prunus persica	*Meloidogyne incognita*	S_0	Hashmi *et al.*, 1995
Sorghum bicolor	*Spodoptera frugiperda*	S_3*	Duncan*et al.*, 1991a; Isenhour *et al.*, 1991
Triticum aestivum	*Diuraphis noxia*	S_2	Zemetra *et al.*, 1993

*Released cultivar.

Biochemical markers, such as accumulation of proline, have been successfully exploited in the *in-vitro* selection of frost-tolerant variants (van Swaaij *et al.*, 1986). Dörffling *et al.* (1993) have selected proline-accumulating cell lines from calli treated with hydroxyproline, and Duncan and Widholm (1987) succeeded in isolating cold-tolerant, proline-accumulating variants of corn by challenging the callus with ABA and by adding mannitol to the medium. However, more ingenious selection methods have been developed. Cryoselected callus led to the isolation of freeze-tolerant wheat. This technique involved the immersion of non-cryoprotected calli in liquid nitrogen (Kendall *et al.*, 1990). The trait was transmitted to the regenerants and to their progenies. However, the callus resulted to be more tolerant than the regenerated plants, and this implied that more factors are involved in the achievement of cold hardiness. In Table 7, more examples of *in-vitro* selections with increased germinability at low temperatures and of other cold-tolerant selections are reported.

Among environmental stresses, increased dosage of UV radiation is causing concern. Levall and Bornman (1993) investigated the possibility of selecting UV-B-tolerant sugarbeet mutants *in vitro*. More examples of the vast possibilities offered by *in-vitro* techniques are possible. For *in-vitro* selection for animal-resistant mutants, three examples are given (Table 7). Zemetra *et al.* (1993) were able to select insect-resistant wheat calli by adding extract of the aphid *Diuraphis noxia* to the medium. Progenies in S_1 and S_2 showed improved resistance. Duncan *et al.* (1991a) and Hashmi *et al.* (1995) identified somaclones of sorghum resistant to fall armyworm and of peach with increased levels of resistance to the nematode *Meloidogyne incognita*, respectively.

Concluding Remarks

Somaclones selected *in-vitro* potentially should have offered greater potential for the isolation of disease and stress-resistant plants, since no other tool available to plant breeders allows them to handle millions of cells, all potential plants, in a confined space and under controlled environmental conditions. However, this has not been the case and despite the large number of successful *in-vitro* selection reports only a few cultivars have arisen from tissue culture. The possibility of selecting only those cells among others that carry the desired trait depends on the use of the proper selective agent. As for adaptation to stress-causing environments, under complex biochemical control, it will become necessary to break the linkage between reduced growth and the adaptation process itself. It is questionable how effective *in-vitro* selection is in activating a possible specific metabolic process, capable of enhancing the fitness of cells under stress, without limiting cell growth.

The rapidity and ease of *in-vitro* selection depends on the knowledge of techniques of tissue culture, pathology and physiology. By coupling these different disciplines the plant breeder will be able to devise the correct *in-vitro* selection protocol. Working *in vitro* means controlling all parameters of the environment in

which plants are grown. The desired results are dependent on the use of proper genotype, type of tissue culture and selective agents.

Environmental stresses are easily reproducible under laboratory conditions, enabling selection, but when dealing with pathogens the selective agent becomes more problematic. Some pathogens produce host-specific toxins and these are the best for isolating disease-resistant variants. Most pathogens do not produce any well-defined toxins, therefore any metabolite that is thought to play a role in disease development could possibly increase partial resistance. This is probably the case with fusaric acid, a metabolite produced by a number of *Fusarium* species.

Somacolonal variation has found its greatest application in plant improvement of ornamental species and in *in-vitro* selection for environmental stresses. It has been used to broaden the genetic bases of a vast array of crops. The ultimate intent should enable the extension of their cultivation into environments which were previously not suitable for them. In most cases the selected traits were under the control of a single; dominant gene. Breeding approaches should combine both conventional and *in-vitro* techniques to realize the full value of *in-vitro* selection. What concerns the selection fro stress tolerance, resistance to stresses, results from rare mutations in few major genes. In the future the ability to select useful mutants *in vitro* may be coupled with molecular techniques, and useful identified somaclones may also be exploited in different crop plants.

The aim of this review is to highlight the most significant examples of *in-vitro* selection for resistance to biotic and abiotic stresses. The main object of this chapter was to focus on disease resistance. In the tables all reports which mentioned the regeneration of tolerant or resistant plants were included, whereas for the abiotic stresses only reports that verified the true genetic nature of the resistance were listed.

In-vitro selection provides a fast method of developing plants with improved traits, by exploiting somaclonal variation occurring *in-vitro.* The evaluation of the selected somaclones is the most problematic element of a selection process. The same quantitative changes that are observable during the *in-vitro* selection may not necessarily be recorded under field trials, in which the environmental impact is more relevant. Frequently, studies that report isolation of a somaclone with increased disease resistance fail to produce evidence of the stability and heritability of the trait. There are different explanations for this phenomenon which may be attribute to: (i) epigenetic changes; (ii) chimaeric nature of the plant material; (iii) the use of non-specific toxins; (iv) the complexity of the trait, e.g. salinity or drought stresses difficult to select based on one of the many components; (v) the failure of cells in differentiated form to express the trait in the regenerated plants and progenies. There cannot be any easy answer to these questions, but the more somaclonal variation is applied to crop improvement the more this technique will prove its full potential.

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11. Somaclonal Variation in Crop Improvement

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Introduction

Deterioration of the environment is increasing the levels of abiotic and biotic stress on plant growth. Further expansion of cultivated land has reached a limit. Rapid industrialization and increase in population are causing environmental changes such as depletion of the atmospheric ozone layer, acid rain, erratic weather, insect and pest problems, diseases, global warming, and increase in the ultraviolet-B (UV-B) radiation level. The adverse environmental impact is gradually leading to reduction in crop production and poses a serious threat to sustained food production. Plant breeders are faced with the challenge to enhance food production for the ever-increasing human population despite the deteriorating environment. So far, conventional plant breeding and crop management practices have enhanced crop production. New approaches and tools are now available to assist plant breeders to improve crops. Plant biotechnology offers new possibilities to sustain agricultural production by offering new opportunities for creating and utilizing genetic variation.

The availability of genetic variation is the basis of breeding new cultivars. In many crops, genetic variation can be obtained through sexual recombination of genes from which desired genotypes are selected. Parasexual techniques of induced mutagenesis, tissue culture and molecular genetics can be used to complement recombination to generate variation. Mutation techniques, in which chemical and physical mutagens have been widely used to induce variation in plants, are already well-established for crop improvement. Cell and tissue culture techniques and molecular methods are more recently available to generate variation in plant improvement. In this chapter the role of tissue culture-derived variation, *somaclonal variation*, in crop improvement is discussed.

Somaclonal Variation

In many species, plants can be regenerated from various cells and tissues, and the regenerated plants often show variation. Somaclonal variation originates in plants obtained through tissue culture (Larkin and Scowcroft, 1981). It includes variation that may result from culture of protoplasts (protoclonal), anthers or microspores (gametoclonal), callus (calliclonal), apical meristems (mericlonal), and leaf and

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stem tissues (somaclonal). Before the generic term 'somaclonal variation' was coined (Larkin and Scowcroft, 1981), variation observed among plants derived from tissue culture was variously described as 'parasexual' (Ahloowalia, 1976), 'pheno-variant' (Sibi, 1976), 'calliclonal' (Skirvin and Janick, 1976), and 'protoclonal' (Shepard, 1981). Somaclonal variation (SCV) may result in genetically stable and useful genotypes (Hammerschlag, 1992; Skirvin *et al.*, 1993; Maluszynski *et al.*, 1995; Jain, 1997a,b; Jain *et al.*, 1997a,b), and in many ways mimics induced mutations. However, not all mutations are expressed such that only a few can be observed as phenotypic and/or cytogenetic changes in the regenerated plants (Remotti, 1997). Such changes, either induced or spontaneous, may provide novel variation in plant breeding programmes and compensate for the narrow genetic base in many crop plants.

Origin of Somaclonal Variation

Many mechanisms have been suggested which lead to somaclonal variation. These include: (1) point mutations induced by various chemicals in the medium; (2) changes in chromosome number and structure; (3) activation of transposons; (4) methylation of DNA; (5) changes in plastid DNA, (6) changes in mitochondrial DNA; (7) segregation of existing chimaeral tissues; and (8) non-specified interactions leading to changes in gene expression, i.e. epigenetic variation that may result from microenvironmental conditions in tissue culture – such variation is generally unstable and non-transmissible. Cytokinin habituation and chilling resistance are examples of epigenetic variation (Hammerschlag, 1992).

The extent and frequency of somaclonal variation is strongly affected by genotype, explant source, culture medium, age of the donor plants and duration of culture. Gonzalez *et al.* (1996) suggested that the source of explant, the type of callus (morphogenic vs. non-morphogenic) and duration of culture was more important than the genotype in determining chromosomal stability of barley cultures. Bebeli *et al.* (1993) showed heritable variation in regenerated rye plants obtained by *in-vitro* culture of immature embryos; such variation was more pronounced in lines lacking telomeric heterochromatin.

Duration of callus culture has a marked effect on the frequency of somaclonal variation. Reduction or even loss of regeneration ability has been observed in many undifferentiated cell cultures. Nehra *et al.* (1990) showed that strawberry callus derived from *in-vitro* cultured leaves completely lost its regeneration capacity after 24 weeks; this loss was associated with the formation of cells with abnormal DNA content. Infante *et al.* (1996) obtained stable cell suspensions of diploid *Fragaria vesca monophylla* that could regenerate even after 2 years from unorganized cell cultures; there was variation in shoot regeneration capability between different cultures. Plants regenerated via somatic embryogenesis can produce true-to-type progeny and minimize variation (Jain *et al.*, 1995). Changes in tissue culture conditions can influence the frequency of variation (Remotti, 1998).

Genetic Analysis of Somaclonal Variation

Many somaclonal variants have been investigated for chromosomal stability. Polyploids, aneuploids, structural changes including chromosomal deletions, inversions, and translocations have been found among plants regenerated from callus culture (Ahloowalia, 1986). However, few somaclones have been investigated for transmission and genetic analysis. In wheat, reduced plant height, multi-tillering, branching from nodes, reduced spike length and grain number have been reported (Ahloowalia and Sherington, 1985; Cheng *et al.*, 1990). Spike shape variants (nodal branching) in wheat were not transmitted to subsequent generations. Likewise, bisexual tassels in maize observed in SC1 were not transmitted to the SC2 (Ahloowalia, unpublished). On the other hand, 13 variants among 230 regenerated plants in tomato were found to be due to single gene mutations, and involved both recessive and dominant changes (Evans and Sharp, 1983).

Somaclonal variation is undesirable for clonal propagation of woody and ornamental plants and transgenic plants or in large-scale mass propagation (Heinze and Schmidt, 1995). The economic disaster can be enormous as a result of undesirable variation in forest trees and other woody plants, since they have long life cycles. It is important to maintain genetic stability in tissue culture-derived plant materials meant for reforestation.

Some genetic changes cannot be observed at the morphological or physiological level because the structural difference in the gene product may not alter its biological activity sufficiently to produce an altered phenotype. Neale *et al.* (1992) suggested the use of molecular markers, such as Randomly Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) as new genetic markers in forest tree improvement, for estimating genetic diversity in breeding populations and for germplasm identification. 'Silent mutations' at morphological and physiological levels are significant since they allow an estimation of the frequency of genomic change as a result of *in-vitro* culture (Sabir *et al.*, 1992). Isozyme markers have proven useful to detect somaclonal variation among regenerants in apple stocks (Martelli *et al.*, 1993). Shenoy and Vasil (1992) analysed somatic embryo-derived Napier grass with several isozymes, but did not find variation among the regenerants, suggesting uniform material for clonal propagation and genetic transformation. Similarly, regenerated plants via somatic embryogenesis in Norway spruce, *Picea abies* (Heinze and Schmidt, 1995; Fourre *et al.*, 1997), *Picea mariana* (Isabel *et al.*, 1993) and in other woody plants have been shown to be clonally identical (Haque *et al.*, 1992) by RAPD and RFLP (Merkle *et al.*, 1988) markers. Taylor *et al.* (1995) observed few RAPD polymorphisms in sugarcane plants regenerated from embryogenic cultures, indicating infrequent genetic change during tissue culture. Rani *et al.* (1995) identified somaclones of *Populus deltoides* using RAPDs. Six of 23 plants propagated from a single clone could be distinguished from the others and field-grown plants by 13 polymorphisms using five primers. These results indicate that

a single somatic mutation had been detected which preceded the cell division and resulted in cells contributing to the six identical variants. There are several forms of DNA variation that RAPD analysis is unlikely to detect, including rare point mutations and duplications of genes or chromosomes. Some of these variations, however, are hardly detectable by current methods of DNA analysis (Fourre *et al.*, 1997). The method of Amplified Fragment Length Polymorphism (AFLP) generates comparatively more markers and may allow the identification of relatively low levels of somaclonal variation (Thomas *et al.*, 1995).

Fourre *et al.* (1997) analysed four embryogenic clones of Norway spruce, that were subcultured over several years to determine the maturity and quality of somatic embryos. A wide range of intraclonal quantitative and qualitative variability was observed, such as immature embryos with diffuse organization, complete or part albino mature embryos. The cytological analysis revealed, for the first time in this species, three types of mutations in somatic seedlings: one totally trisomic and two chimaeras with trisomic buds and diploid roots. Also, tetraploid and mixoploid embryogenic masses were detected. The RAPD analysis did not show any variation despite the large sample of DNA and primers used. Fourre *et al.* (1997) suggested that the use of molecular markers (RFLPs, RAPDs) or isozymes to assess the genetic stability of an *in-vitro* production system is by itself insufficient, and the morphological and cytological approach appears to be a valuable complementary tool.

A high frequency of somaclonal variation for morphological characters has been reported in sandalwood (*Santalum album*) plants regenerated from somatic embryos that had been differentiated *in vitro* from embryogenic callus initiated from stem segments (Rao *et al.*, 1984). Because the frequency of somaclonal variation appears to increase with duration of *in-vitro* culture, especially in callus or cell suspension cultures (Deverno, 1995), cryopreservation of somatic embryogenic cultures could be used in lieu of continuous subculturing. The maintenance of genetic fidelity of tissue culture-derived regenerated plants is essential to preserve the genetic integrity of elite plant material.

Mutagenesis

Induced mutations using gamma irradiation or chemical mutagens may yield useful genetic changes – high yield, flower colour, disease resistance, early maturity, etc. – in crop plants (Micke *et al.*, 1990; Crino *et al.*, 1994). The 1996 annual report of the International Atomic Energy Agency (IAEA), Vienna, states that by 1995 nearly 1700 mutant varieties had been officially released in 52 countries. A high-yielding 'naked grain' barley has been released in the Altiplano, Peru, by selecting mutants from gamma-irradiated seeds. This region is more than 3600 m above sea level, and has a short growing season. The new cultivar exhibits improved adaptability to this stress-prone area, and is well accepted by the consumers. Maluszynski *et al.* (1995) pointed out that, even though most induced mutations are recessive and deleterious, mutation breeding has contributed significantly to plant improvement worldwide. For example, cultivars of salt-

tolerant rice in China, high-yielding cotton in Pakistan, and heat-tolerant wheat in India have been released.

Genetic variation can be enhanced by combining mutagenesis and tissue culture for crop improvement. Jain (1997b) irradiated axillary buds with gamma rays, excised from *in-vitro*-grown strawberry plants; 5% survived selection pressure to *Phytophthora cactorum* crude extract and the resulting plants were able to withstand water-holding for 5–6 days. It seemed that pathogen-related proteins may be responsible for both drought and disease tolerance. However, Eddo Rugini (personal communication) demonstrated an increase in endogenous phenols of strawberry selected *in vitro* against *Rhizoctonia fragarie* toxin, and the selected shoots also showed resistance to *Botrytis cinera.* Banerjee and Kalloo (1989) observed high total phenol content in disease and pest-resistant wild-type tomato (*Lycopersicon hirsutum* f. *glabratum*) lines compared to susceptible cultivated tomato (*Lycopersicon esculentum*) and suggested that this parameter could be used for selection of disease- and pest-resistant lines in cultivated tomato. A comparative study of SCV versus induced mutagenesis indicated that the two sources of variability differed in their effect, changing the pattern of segregation in *Lycopersicon esculentum* (Gavazzi *et al.*, 1987) and *Brassica napus* (Jain and Newton, 1988, 1989). Cheng *et al.* (1990) used gamma rays, sodium azide and ethyl methane sulphonate mutagens, and showed that mutagen-induced gain in terms of reduction in plant height, fertility and spike length was not outstanding in regenerated plants compared with the untreated control. Furthermore, somaclones following mutagenesis can be at least partially independent and synergistic in nature, hence providing more selection opportunities for plant improvement. In spite of some promising results, further efforts are still needed to develop procedures for *in-vitro* mutagenesis to create more useful variation along with somaclonal variation. Table 1 lists agronomically important mutants induced in crop plants. There is not much information available on mutagenesis in forest trees.

Table 1. Agronomically important induced mutants in plants (Jain, 1997a)

Plant	Mutagenic treatment	Trait improved
**Avena sativa*	Gamma irradiation	Rust resistance
**Brassica juncea*	Gamma rays	Earliness
**Chrysanthemum sp.*	Gamma rays	Flower colour
**Glycine max L.*	Gamma rays	Earliness, seed colour, disease resistance
**Gossypium sp.*	Gamma rays	Semi-dwarf
**Oryza sativa*	Gamma rays	Earliness, disease resistance, lodging resistance, salt tolerance, cold tolerance, grain quality
**Rosa sp.*	Gamma rays	Flower colour
**Sesamum indicum*	Gamma rays	Oil quality and yield, protein
**Vigna radiata*	Gamma rays	Salt/acid tolerance, yield
Trititcum aestivum	Gamma rays	Heat tolerance
Brassica napus	Ethyl methane sulfonate	Yellow seed
Fragaria × *ananassa*	Gamma rays	Disease resistance, drought resistance

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In-vitro Selection

In-vitro selection can be exploited to select agronomically desirable somaclones for tolerance to drought, disease, salinity, cold and freezing under laboratory conditions. Such selection systems are fast and easy, and may save several years of work required in conventional breeding. Furthermore, this process minimizes the influence of environment and can be enhanced depending upon the needs. However, it is essential to field test the regenerated plants for genetic stability as the selection agent itself may induce a different array of unexpected somaclonal variation (Alicchio *et al.*, 1984). Several agronomically important somaclones have been selected *in vitro.*

Stability of Somaclonal Variants

Somaclonal variation can be used for breeding new cultivars only if it is heritable and genetically stable. Most somaclonal variation has been of limited value in plant breeding because: (1) it is unpredictable in nature, and may be heritable or non-heritable, being transient or 'epigenetic'; (2) somaclonal variation cannot be obtained for all traits; (3) many characters change in the negative direction; (4) much variation is not novel, and (5) subsequent breeding and field evaluation is still needed once a desirable variant has been found. However, somaclonal variation does provide some advantages: (1) variants can occur for agronomic traits; (2) variation often occurs at a high frequency; (3) some variants may be novel and have not been obtained by conventional breeding; (4) in some cases, *in-vitro* selection may allow isolation of genotypes tolerant to abiotic and biotic stresses. In potato, selected somaclones, derived from tuber discs, showed desirable alterations in several traits such as yield, tuber number and shape, and vigour. These changes were stable over more than two consecutive asexual propagation cycles (Rietveld *et al.*, 1991). Stephens *et al.* (1991) found minor differences that were mostly beneficial in self-pollinated soybean progenies following regeneration through organogenesis; in comparison with the parent, significant variation was found for maturity, lodging, height, seed protein and oil, but not for seed quality, seed weight, and seed yield. These examples demonstrate that stable genetic variation can persist through both sexual and asexual cycles following a tissue culture phase. Such variants may be useful for breeding new cultivars.

Somaclonal Variation and Floriculture

Propagation of many ornamental plants is big business. Tissue cultured ornamental plants often exhibit somaclonal variation as altered plant morphology, leaf morphology, flower colour and shape or leaf variegation. Jain (1993a,b) reported somaclonal variation for flower size, plant height, plant morphology, and number of flowers per plant in *Begonia* × *elatior* plants regenerated from leaf-disc callus.

Similar variation was observed in plants regenerated from leaf discs of *Saintpaulia ionantha* L. without callus phase. However, flower colour variation was not found in either species (Jain, 1993a,b). Wolff *et al.* (1986) reported variation for days to flowering, flowers per peduncle, peduncle length and leaf area among the progeny of *Streptocarpus* × *hybridus* that had been regenerated from anther-derived callus. Flower colour variation has been reported in tissue culture-derived plants of carnation (Silvy and Mitteau, 1986), chrysanthemum (Khalid *et al.*, 1989), and gerbera (Buiatti and Gimelli, 1993). Selected somaclones can be further micropropagated to establish their stability in subsequent generations (Jain, 1997c). Jain (1993a,b) observed that selected somaclones of *Begonia* and *Saintpaulia* did not show variation for number of flowers per plant in two subsequent generations. It seems that somaclonal variation can be exploited commercially in ornamental plants.

History and Application of Somaclonal Variation in the Solanaceae

Because of their general amenability to tissue culture techniques, Solanaceous plants have held a unique position in the study of regeneration phenomena and variation among regenerated plants. Protocols for protoplast-to-plant were initially developed for tobacco (Nagata and Takebe, 1971) and shortly thereafter for potato (Shepard and Totten, 1977) and tomato (Morgan and Cocking, 1982). Therefore, these important crop species have served as model plants for the application of protoplast technology as well as for documentation of the initially unexpected and now mundane somaclonal variation that accompanies application of this technology. The causes that underlie somaclonal variation have also been more intensively studied in Solanaceous plants than in any other. A review of somaclonal variation in the Solanaceae follows. More comprehensive reviews of literature of the 1980s for individual Solanaceae have been published by Karp (1990) for potato, Buiatti and Morpurgo (1990) for tomato, Alicchio (1990) for eggplant, and Prat *et al.* (1990) for tobacco.

Shepard *et al.* (1980) first described extensive somaclonal variation in a population of more than 10 000 protoplast-derived clones of the commercially important potato cv. Russet Burbank. They reported many types of morphological variants as well as somaclones that exhibited more disease resistance than the parental cultivar. They concluded cautiously that somaclones with an improved character may well exhibit deficiencies in other agronomic characters, and therefore the results were preliminary with regard to the application of somaclonal variation to varietal improvement. In a complementary study of somaclones preselected for relatively normal appearance, Secor and Shepard (1981) documented statistically significant differences for 22 of 35 characters examined; each of 65 somaclones differed from the parent cultivar for at least one trait. Thomas *et al.* (1982) confirmed extensive morphological variation among protoclones of cv. Maris Bard, even among plants derived from different shoots of the same callus colony. Agronomic evaluation of 33 protoclones of cv. Crystal revealed

that none had greater tuber yield than the parent cultivar; however, improved resistance to tuber bruising, enhanced chip colour and elevated resistance to tuber soft rot were reported in some of the somaclones (Taylor *et al.*, 1993). The authors concluded that somaclonal variation was useful to select incrementally for cultivar enhancement. However, with Russet Burbank, Secor (personal communication) has given up on somaclonal variation for cultivar enhancement after many years of field trials of selected protoclones. He believes that the cultivar is too plastic, leading to somaclones that exhibit too much undesirable variation in agronomic traits even when a single trait may be improved.

Tomato has been considerably more recalcitrant to tissue culture techniques than other Solanaceous plants. Therefore, studies of somaclonal variation have been more concerned with plants regenerated from brief callus phases rather than from mesophyll protoplasts as the latter procedure has been restricted to only a few cultivars (Niedz *et al.*, 1985). Sibi (1982) observed heritable variation in 10 of 30 families of tomato somaclones regenerated from cotyledonary callus. Gavazzi *et al.* (1987) reported that the spectrum of mutations induced during *in-vitro* regeneration from cotyledonary callus differed from that after ethyl methane sulphonate treatment of seeds or pollen and that the somaclonal variants were more abundant. A similar trend of higher mutant frequencies after ethyl methane sulphonate treatment compared to somaclones from callus cultures was found by van den Bulk *et al.* (1990). The source of tissue (leaf, cotyledon or hypocotyl) used to induce callus was found to influence the frequency of polyploid plants regenerated, and this was correlated with the percentage of polyploid cells present in the explants. Progenies of 279 somaclones of cv. Moneymaker were subsequently screened for resistance to bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) without finding any resistance (van den Bulk *et al.*, 1991). The authors questioned the potential of tissue culture as a source of novel disease resistance. On the other hand, Barden *et al.* (1986) and Smith and Murakishi (1993) identified tomato somaclones resistant to tomato mosaic virus and characterized the resistance as due to restricted virus movement. Montagno *et al.* (1989) reported less genetically inherited variation after callus induction and regeneration from cotyledons compared to similar cultures that had been gamma irradiated. Wisman *et al.* (1993) reported the appearance of a paramutagenic allele that had arisen among somaclonal variants of cv. Moneymaker after regeneration from callus. Paramutation was defined as the imposition of expression of a mutant allele on a wild-type allele in heterozygous condition.

Variation for chromosome number has frequently been observed among potato somaclones (Pijnacker and Sree Ramulu, 1990). Sree Ramulu *et al.* (1983) reported that most of the grossly abnormal protoclones of cv. Bintje were octoploid or mixoploid. Sree Ramulu *et al.* (1986) found that the initial ploidy of the plant influenced the degree of polyploidization that occurred during protoplast isolation and culture; none of the regenerants from a diploid potato clone had retained the diploid chromosome number – all were tetraploid or aneuploid – whereas nearly 40% of the regenerants from the tetraploid cv. Bintje had retained the tetraploid chromosome number. However, a higher frequency of tetraploid protoclones of

Bintje were morphologically abnormal compared to the tetraploid protoclones of the diploid genotype. Zhila *et al.* (1987) reported only 15% normal tetraploid plants among protoclones of tetraploid cv. Zarevo with the remaining plants mostly aneuploid and mixoploid. Creissen and Karp (1985) reported 57% normal tetraploid regenerants from protoplasts of the potato cv. Majestic; however, even among the phenotypically normal regenerants, three plants were found that exhibited chromosomal structural rearrangements. When a monoploid potato clone was used as a source of protoplasts, most regenerated plants were tetraploid, octoploid or aneuploid – no monoploid or diploid protoclones were identified (Sree Ramulu *et al.*, 1989). Sadanandam (1991) characterized a synaptic mutant among protoclones of a dihaploid potato clone that had been exposed to ethyl methyl sulphonate for 1 h after protoplast isolation.

The duration of the culture phase and the type of culture from which protoplasts have been isolated can affect the frequency of abnormal regenerants. Sree Ramulu *et al.* (1986) found that protoclones from cell suspension cultures of a diploid potato clone exhibited more somaclonal variation than those from shoot cultures. Burg *et al.* (1989) found that the frequency of variants from cv. Bintje was greater among the late regenerants (102 days after protoplast isolation) than from the early regenerants (82 days after isolation).

A major reason for ploidy variation among protoclones is genetic instability during culture. Sree Ramulu *et al.* (1984) reported that endoreduplication, polyploidization and aneuploidy occurred during the initial stages of protoplast development and callus induction, and continued during callus growth. Within just a few days of culture initiation from protoplasts of a tetraploid potato clone, Carlberg *et al.* (1984) found high frequencies of endopolyploid cells. Owen *et al.* (1988) demonstrated that the frequency of endopolyploidization among somatic cells within monoploid potato genotypes was under genetic control; indeed, most of the cells in a monoploid plant were at the diploid level before protoplast isolation. Therefore, many protoplasts start as polyploid cells. Another reason for frequent polyploidization and aneuploidization during protoplast culture is acytokinesis, i.e. nuclear division without cell division, leading to polynucleate cells (van Everdink and Pijnacker, 1994). Such binucleate cells accounted for approximately 20% of the total 5 days after protoplast isolation. Spindle interactions or fusion in binucleate cells were thought to result in abnormal chromosome numbers after cell division. Sree Ramulu *et al.* (1985) found that monoploid genotypes demonstrated a more rapid rate of polyploidization than diploid or tetraploid genotypes. Even before protoplasts were isolated, Wolter *et al.* (1994) found that a low frequency of aneuploid cells was evident in root tip metaphases of tomato and potato plantlets that were used in fusion experiments.

The first characterization of somaclonal variation at the molecular level was done on potato protoclones (Landsmann and Uhrig, 1985); two of the 12 derivatives of plants regenerated from protoplasts exhibited 70% reduction in 25 S ribosomal RNA genes by Southern hybridization of restricted DNA with a probe homologous to 25S rRNA. Lindeque *et al.* (1991) used protein-DNA binding to characterize callus-derived plants of *S. tuberosum* cv. BP1, and reported that the

amount of DNA binding was correlated to the vigour of the somaclones. Potter and Jones (1991) compared restriction patterns of micropropagated potato plants with those of plants regenerated from leaf callus. No variation was found among 128 micropropagated plants. However, six of the 46 plants regenerated after a brief callus phase exhibited aberrant RFLP patterns. In tomato, Rus-Kortekaas *et al.* (1994) failed to detect any differences in either RAPDs or microsatellite patterns of 12 calluses and 12 second-generation regenerants compared to the original cultivar. Smulders *et al.* (1995) found DNA methylation changes in callus culture, some of which persisted in regenerated plants of tomato and were partly transmitted to the progeny of regenerated plants.

Another unexpected effect of somaclonal variation that has been demonstrated in Solanaceous plants has been the observation of altered recombination distances during meiosis. Sibi *et al.* (1984) reported an increase in genetic distance between marker loci for approximately half of the tomato plants that had been regenerated from cotyledonary callus. Compton and Veilleux (1991) later showed that altered genetic recombination in tomato could occur even after what is considered a more benign tissue culture procedure of micropropagation. Lentini *et al.* (1990) exploited the potential of altered segregation following callus culture by regenerating interspecific potato hybrids from callus, with the result that undesirable linkages between trichome traits conferring insect resistance and poor agronomic performance were broken. They concluded that a period of callus culture followed by plant regeneration may aid in the introgression of desirable traits from wild species into crop plants. Burgutin *et al.* (1994) examined somaclones regenerated from an interspecific somatic hybrid between *S. tuberosum* and *S. chacoense* Bitt. f. *parodii*, and reported evidence of segregation of isozyme markers among the somaclones regenerated from internode callus of the somatic hybrid; the markers that were characteristic of both parents were present in the somatic hybrid.

One additional area in which Solanaceous plants have served as test crops is that of *in-vitro* selection to attempt to direct somaclonal variation. Taylor and Secor (1990) found differential callus longevity when protoplast-derived calluses of potato cv. Crystal were exposed to a suspension of the soft rot organism, *Erwinia carotovora* subsp. *carotovora.* Cell lines resistant to hydroxyproline, an amino acid analogue, were selected from leaf callus, and suspension cultures of a diploid potato clone and four of 67 lines were regenerated into plants (van Swaaij *et al.* 1987). Regenerants of the hydroxyproline cell lines exhibited 5–6 times higher resistance to hydroxyproline than the controls. Though variable, leaves and tubers of regenerants generally contained elevated levels of proline and total amino acids. A concomitant increase in frost tolerance of the hydroxyproline-resistant plants was observed; however, the plants also displayed various degrees of undesirable phenotypic variation. Sebastiani *et al.* (1994) selected nodal explants from shoots regenerated from stem explants of potato cv. Desiree to fungal filtrates of the wilt-inducing pathogen, *Verticillium dahliae.* Of 325 regenerated plants, one was found that exhibited resistance equivalent to a resistant control and showed marked improvement compared with the infected Desiree control.

Various other traits of the resistant *in-vitro* selection line were examined, and did not differ from Desiree.

There have been probably more somaclones of Solanaceous plants examined for field performance than of any other plant family. However, no cultivar releases have been made as far as we know. For genetic engineering, somaclonal variation is regarded as a hindrance because the only desired difference between the starting plant material and the product is the expression of transgene. Somaclonal variation can be compared with mutation breeding in that it is a source of variation some of which may be useful, and with *in-vitro* selection, can be targeted for a particular trait. However, the target is not achieved with the frequent deficiencies in agronomic performance.

Use of Somaclonal Variation in Plant Breeding

Only a few somaclones have been of direct value without further breeding. Most somaclones have been used as a germplasm source. These include somaclonal variants for high solids in tomato. High-yielding inbred lines of maize obtained through somaclonal variation have been used in hybrid seed production in the USA. Two high-yielding somaclones in *Brassica* (BIO-902 and BIO-YSR) that outperformed the parental cultivar 'Varuna' in seed yield have been recently reported in India. There is a distinct advantage of somaclonal variants in vegetatively propagated plants where conventional breeding is difficult or impossible through sexual crossing. The frequency of somaclonal variation in many plant species is low and results in mostly undesirable changes, especially in cereals, and is, therefore, of little use in crop improvement (Ahloowalia, 1986). Maddock (1986) also observed low frequency of somaclonal variation in wheat.

Conclusions

Plant tissue culture has a vast potential to improve cultivated plants. Somaclonal variation (SCV) and induced mutations have resulted in the production of new genotypes with a limited change in the original genome. As a source of variation, SCV mimics induced mutations. *In-vitro* selection is a quick approach to produce plants with resistance to environmental abiotic and biotic stresses. Many initially apparent somaclones arise as a result of epigenetic change and revert back to the original phenotype in the subsequent sexual or clonal generations. Hence, it is essential to test the genetic stability of selected somaclones before their incorporation in plant breeding programmes. Molecular markers such as RFLPs, AFLPs, and RAPDs facilitate the identification of genetically stable vs. epigenetic somaclones. In cereals, the frequency of somaclonal variation appears to be comparatively low, and hence of limited value in grain improvement. Therefore, it is desirable to combine tissue culture and mutagenesis to obtain a wide range of variation as a reliable tool for plant breeding.

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12. Field Performance of Banana Micropropagules and Somaclones

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Introduction

Banana and plantain (*Musa* spp. L.) are giant perennial herbs that thrive in the humid tropics and subtropics. The cultivated banana is mostly triploid ($2n = 3x = 33$), although diploid and tetraploid cultivars occur in much lower numbers. Its fruit provides one of the major commodities in international trade, but is far more important as a starchy staple food in local economies (Stover and Simmonds, 1987). However, production is threatened by pest and disease pressure, which have been increasing during the past 20 years. Most alarming has been the spread of more virulent forms of the fungal diseases black sigatoka leaf spot (*Mycosphaerella fijiensis* Morelet) and fusarium wilt (*Fusarium oxysporum* Schlecht. f.sp. *cubense* (E.F. Smith) Snyd. & Hans.). In response to these production constraints, efforts aimed at the genetic improvement of *Musa* have gained renewed interest (Persley and De Langhe, 1987; Rowe and Rosales, 1996; Vuylsteke *et al.*, 1993a, 1997). *Musa* genetic improvement by conventional hybridization is, however, complex and difficult as it is burdened with obstacles typical of polyploid, vegetatively propagated crops, among which the trisomic pattern of gene inheritance, low seed fertility, and slow propagation are most conspicuous. Hence, biotechnology has been investigated for *Musa* germplasm handling and improvement (INIBAP, 1993; Novak, 1992; Vuylsteke, 1989).

Application of *in-vitro* culture techniques has greatly improved *Musa* germplasm handling for the purposes of clonal propagation, uniform production, and breeding. Micropropagation using shoot-tip culture has played a key role in plantain and banana improvement programmes worldwide, e.g., those of the Fundacion Hondurena de Investigacion Agricola (FHIA) in Honduras (Rowe and Rosales, 1996), of the International Institute of Tropical Agriculture (IITA) in Nigeria (Vuylsteke *et al.*, 1993a, 1997), and others. Valuable *Musa* germplasm, including sources of disease and pest resistance, has been exchanged among all continents using *in-vitro* cultures (Van den Houwe and Jones, 1994; Vuylsteke *et al.*, 1990a) and is conserved *in-vitro* (Panis *et al.*, 1990; Vuylsteke, 1989). Large numbers of micropropagated plants have been produced for rapid establishment of crossing blocks and for international, multi-site hybrid evaluation trials (Van den Houwe and Jones, 1994; Vuylsteke *et al.*, 1990b, 1993b, 1997).

Plant tissue culture techniques are frequently used for the propagation of horticultural crops, such as the banana, because of the advantages they confer, *viz.*: (a) higher rates of multiplication, (b) production of clean or disease-free

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planting material, and (c) the small amount of space required to maintain or multiply large numbers of plants. Development of a shoot-tip culture technique for *in-vitro* micropropagation of *Musa* has thus received a lot of attention during the past two decades and has become well established (Cronauer and Krikorian, 1984; Hwang *et al.*, 1984; Israeli *et al.*, 1995; Smith and Drew, 1990a; Vuylsteke and De Langhe, 1985; Vuylsteke, 1989). Micropropagation of banana has also attracted large-scale commercial interest, particularly for the large dessert banana plantations (Oglesby and Griffis, 1986; Smith and Drew, 1990a).

Some plants regenerated from *in-vitro* culture have exhibited various morphological and biochemical variations due to genetic changes, which Larkin and Scowcroft (1981) termed somaclonal variation. Somaclonal variation is ubiquitous in *Musa*, like in other plants. *Musa* plants produced by shoot-tip culture have shown somaclonal variation rates of 0–70%, mainly depending on the genotype (Cote *et al.*, 1993; Smith, 1988; Vuylsteke *et al.*, 1991), and this has often been acknowledged as a problem or risk associated with the use of *in-vitro* culture techniques for germplasm handling. Conversely, somaclonal variation has recently generated great interest as a potential source of novel and useful variability. Genetic variability is an essential component of any plant improvement programme. Hence, developing novel methods for producing genetic variability is important, particularly if this variability is lacking. Banana improvement via somaclonal variation thus has been advocated, because conventional breeding is difficult and because the banana is amenable to various tissue culture techniques (Novak, 1992; Ortiz and Vuylsteke, 1996; Smith and Drew, 1990a). For instance, production through somaclonal variation of a useful mutant which confers disease resistance in an existing cultivar would be highly desirable (Krikorian, 1989).

Frequent use of tissue culture for *Musa* germplasm handling and improvement warrants investigation into the occurrence of somaclonal variation in this genus, including the agronomic evaluation of micropropagated bananas and its somaclonal variants. Qualitative aspects of somaclonal variation in *Musa* have been documented to a reasonable extent (Cote *et al.*, 1993; Israeli *et al.*, 1991; Smith, 1988; Vuylsteke *et al.*, 1991), but quantitative variation has been less characterized. Here, we review reports on the field performance of *in-vitro* propagated plants and somaclonal variants of banana and plantain.

Performance of Micropropagules

Several authors have documented the field performance of micropropagated bananas, particularly with a strong bias towards cultivars of the 'Cavendish' subgroup (*Musa* spp., AAA group), which constitute the bulk of the international banana trade (Stover and Simmonds, 1987). When compared with bananas propagated by conventional planting material, i.e. suckers, micropropagated banana plants were capable of performing equal or better (Hwang *et al.*, 1984; Smith and Drew, 1990a). In general, micropropagated banana plants established more quickly, grew more vigorously, were taller, had a shorter and more uniform pro-

duction period, and produced higher yields than conventional propagules (Arias and Valverde, 1987; Daniells, 1988; Drew and Smith, 1990; Espino *et al.*, 1992; Hwang *et al.*, 1984; Israeli *et al.*, 1988; Kwa and Ganry, 1990; Robinson *et al.*, 1993; Zamora *et al.*, 1989). Yield gains from the use of *in-vitro* plants reached up to 20% (Table 1), but this was sometimes confined to the plant crop only or was season-dependent in the subtropics (Drew and Smith, 1990; Israeli *et al.*, 1988). Generally, superior performance of micropropagated bananas was due to the fact that such propagules already possessed an active root and shoot system at the time of planting, and were free of most diseases and pests (Drew and Smith, 1990).

Field performance of micropropagated vs. conventionally propagated plantains (*Musa* spp., AAB group) has been reported in two studies (Liu *et al.*, 1989; Vuylsteke and Ortiz, 1996). In both cases, micropropagated plantains grew vigorously and were taller than conventional plants. However, this vigour resulted in significant yield increases of 13–74% only in Puerto Rico (Liu *et al.*, 1989), while Vuylsteke and Ortiz (1996) did not observe the expected higher yield due to severe black sigatoka disease and suboptimal husbandry (Table 1). The latter authors also reported that seed production rates were equal in micropropagated and conventionally propagated plantains.

Results of bananas in Israel (spring planting; Israeli *et al.*, 1988) and plantains in Nigeria (Vuylsteke and Ortiz, 1996) suggest that micropropagated plants of

Table 1. Yield gain of micropropagated over conventional propagules of banana and plantain (*Musa* spp. L.)

Cultivar	Yield gain (%)	Country	Reference
Cavendish bananas (AAA)			
Williams, Grand Nain	20^{**}	South Africa	Robinson *et al.*, 1993
New Guinea Cavendish	16^{**}	Australia	Drew and Smith, 1990
Robusta	12^{NA}	India	Mascarenhas *et al.*, 1983
Unspecified	12^{NA}	Côte d'Ivoire	Kwa and Ganry, 1990
Williams	10^{NA}	Israel (summer)	Israeli *et al.*, 1988
Williams	7^{**}	Australia	Daniells, 1988
Grande Naine	5^{NA}	Costa Rica	Arias and Valverde, 1987
Giant Cavendish	2^{NS}	Taiwan	Hwang *et al.*, 1984
Grand Naine	-18^{NA}	Israel (spring)	Israeli *et al.*, 1988
Plantains (AAB)			
Congo Enano	74^{**}	Puerto Rico	Liu *et al.*, 1989
Maricongo	30^{NS}	Puerto Rico	Liu *et al.*, 1989
Enano Comun	16^{NS}	Puerto Rico	Liu *et al.*, 1989
Congo	13^{NS}	Puerto Rico	Liu *et al.*, 1989
Agbagba	6^{NS}	Nigeria	Vuylsteke and Oritz, 1996
Cooking banana (ABB)			
Cardaba	12^{NS}	Philippines	Espina *et al.*, 1992

^{NS}, *, **,Non-significant or significant at $p \leq 0.05$ or 0.01, respectively; ^{NA}, mean separation not available.

Musa do not manifest consistently superior horticultural performance compared to sucker-propagated plants. It seems that *in-vitro* plants should be regarded as one component of good crop husbandry, not as a guarantee to achieve maximum yields. Micropropagated plants, in combination with other good cultural practices, such as correct planting time (particularly in the subtropics), fertilizer or manure, and mulch, will establish quickly, have vigorous vegetative growth, and produce high yields of good-quality fruit (Robinson *et al.*, 1993; Vuylsteke and Ortiz, 1996). Furthermore, the improved phytosanitary status of *in-vitro* plants makes them the planting material of choice to reduce the risk of pest and disease introduction in new plantings (Hwang *et al.*, 1984) or for establishment of field nursery areas (Smith and Drew, 1990a). These nurseries can then provide clean conventional propagation material for production fields. Roguing of somaclonal variants could also be performed in such nurseries to avoid the occurrence of off-types in production fields at great economic loss to the plantain or banana farmer.

Tissue culture plants thus have great potential to augment the positive effects of other inputs, particularly in high-input agriculture. However, a troublesome characteristic of micropropagated bananas is their increased susceptibility to virus, *Fusarium* wilt and nematodes, when compared to conventional propagules. Several authors reported that cucumber mosaic virus incidence was greater in micropropagated than in conventional bananas (Israeli *et al.*, 1995; Kwa and Ganry, 1990; Sarah *et al.*, 1990). Smith *et al.* (1997) observed that micropropagated plants of the susceptible 'Williams' and resistant 'Goldfinger' showed significantly higher incidence and severity of *Fusarium* wilt (race 4) infection than plants grown from conventional planting material. The greater pest/disease susceptibility of *in-vitro* plants generally disappears as the crop ratoons. The cause and mechanism of this phenomenon are not well understood.

Performance of Somaclonal Variants

Phenotypic variants or off-types are commonly observed in clonal populations of bananas and plantains, but *in-vitro*-produced clones generally show much higher rates of variation than conventionally propagated *Musa* (Drew and Smith, 1990; Smith, 1988; Vuylsteke *et al.*, 1988; Vuylsteke and Ortiz, 1996). The tissue culture-induced variation, referred to as somaclonal variation, mostly mimics naturally occurring variation or produces abnormal phenotypes (Israeli *et al.*, 1991; Smith, 1988; Stover, 1988; Vuylsteke *et al.*, 1991). Phenotypic changes observed in *in-vitro* banana plants may vary from gross abnormalities to very discrete changes in morphology. The most commonly observed morphological changes are dwarfness in the 'Cavendish' bananas and inflorescence variation in the plantains. Changes in leaf, pseudostem and flower sizes, shapes and colours are also common. Except for morphological characterization, there has been limited cytological, biochemical, molecular and genetic analysis of somaclonal variants in *Musa*, although some progress has been made recently. Shepherd and Dos Santos

(1996) described numerical and structural chromosome variation in tissue culture-derived and conventionally propagated banana plants. They concluded that triploid banana is not totally stable in its somatic karyotype, but that *in-vitro* culture can greatly increase the frequency of chromosomal aberrations. Such chromosomal rearrangements are believed to be a major cause of somaclonal variation (Karp and Bright, 1985; Lee and Phillips, 1988). In an attempt to develop early detection methods of the dwarf off-types in the 'Cavendish' bananas, Damasco *et al.* (1996a) found that the dwarf variants were non-responsive to gibberellic acid (GA_3), suggesting a biochemical change in the variants. Sandoval *et al.* (1995) quantified the differences in endogenous GA_3 and GA_{20} levels among normal 'Cavendish' bananas and its dwarf and giant off-types, and suggested that these differences have a genetic origin. Dwarf and giant variants had lower and higher GA content, respectively, than true-to-type plants. Damasco *et al.* (1996b) observed polymorphisms between normal and dwarf 'Cavendish' plants when using random amplified polymorphic DNA (RAPD) primers, revealing genetic change due to somaclonal variation.

Field evaluation of somaclonal variants in micropropagated dessert bananas of the 'Cavendish' subgroup has been reported by several authors (Hwang and Ko, 1987; Israeli *et al.*, 1991; Smith and Drew, 1990b; Stover, 1987, 1988). Variants in plant stature (e.g. dwarfs), foliage, and pseudostem pigmentation were observed. Variants were mostly inferior to the 'Cavendish' clone from which they were derived (Hwang and Ko, 1987; Israeli *et al.*, 1991; Smith and Drew, 1990b; Stover, 1987). Bunch and fruit of the variants were smaller and of little or no commercial value (Table 2). The most common (70–90%) dwarf off-types have been well characterized and appeared stable over several ratoons. Dwarfs were significantly shorter, had smaller fruit, denser hands, and were prone to choking (failure of the bunch to emerge fully from the crown) when minimum temperatures reached 15°C or lower (Israeli *et al.*, 1991; Smith and Drew, 1990b).

Hwang and Ko (1987) identified somaclonal variants of the cultivar Giant Cavendish with putative field resistance to *Fusarium* wilt (race 4), but all had

Table 2. Yield of micropropagated plants of 'Cavendish' banana (*Musa* spp., AAA group) and its somaclonal variants

True-to-type cultivar	Bunch mass (kg)	Bunch mass (kg) of variants		Reference
		'Dwarf'	'Thin leaf'	
Giant Cavendish	29.6	12.0–28.4[†]	–	Hwang, 1988
Grand Nain	32.5	20.0	–	Pool and Irizarry, 1987
Grand Nain	21.7	20.5	–	Israeli *et al.*, 1991
New Guinea Cavendish	36.2	26.2–33.7	38.6	Smith and Drew, 1990b
Williams	25.3	23.5	–	Israeli *et al.*, 1991

[†]Highest yield obtained in an improved selection of the original variant.

inferior agronomic characteristics, including poor yield and low-quality fruit. However, further selection among *in-vitro* or sucker-propagated clones of these resistant variants resulted in the identification of plants with improved traits when compared to the original variant, yet still slightly lower-yielding than the true-to-type cultivar (Table 2) (Hwang, 1988; Hwang *et al.*, 1993). Also, the *Fusarium* wilt resistance of these variants could not always be confirmed when tested in other locations (Israeli *et al.*, 1995), suggesting problems in screening for this disease or the occurrence of genotype-by-environment interaction for this trait. Recently, researchers in Venezuela claimed to have selected a somaclonal variant of 'Cavendish' with resistance to yellow sigatoka (*Mycosphaerella musicola*) and which yielded satisfactorily (Trujillo and Garcia, 1996). These two examples seem to suggest that somaclonal variation from micropropagation of banana may have the potential to confer disease resistance to existing susceptible cultivars. However, it is important not to put too much hope in somaclonal variation for production of disease resistance (Daub, 1986; Krikorian, 1989).

While some work has been done on the characterization of somaclonal variation in plantain (Krikorian *et al.*, 1993; Ramcharan *et al.*, 1987; Sandoval *et al.*, 1991; Vuylsteke *et al.*, 1991), little has been done on the field performance of somaclonal variants of plantain. Variants in inflorescence morphology and foliage have been observed, mostly mimicking natural variation. Ramcharan *et al.* (1987) reported on the field performance of an inflorescence off-type ('French reversion') of each of two 'Horn' plantain clones. The 'French' off-types produced double yields and more fruit (Table 3), but had a longer maturation time than the true-to-type plantains. Vuylsteke *et al.* (1996) provided a comprehensive description of the agronomic performance of four somaclonal variants that were obtained in micropropagated plantings of a common 'False Horn' plantain cultivar. Somaclonal variants were distinguished from true-to-type plants by their variant leaf and inflorescence morphology (Vuylsteke *et al.*, 1988, 1991). In terms of quantitative trait variation, significant variation was observed for plant and fruit maturity, leaf size, yield and its components, but not for leaf number, plant height, or suckering. All off-types differed from true-to-type plantain in yield and bunch characteristics and in fruit maturity, while the two foliage variants also differed in leaf size and plant maturity. Three of the four somaclonal variants were agronomically inferior to the original clone with poor yields due to inflorescence degeneration or abnormal foliage. Only the 'French reversion' variant, which resembled an existing cultivar, outyielded the true-to-type clone (Table 3), but its fruit weight and size were lower and its plant and fruit maturation periods longer, which are less preferred qualities (Vuylsteke *et al.*, 1996). The 'French reversion' variant also showed increased seed fertility (Vuylsteke *et al.*, 1991, 1993b). The lack of significant variation in plant height and in total number of leaves underscored the absence of dwarf or giant variants, which occur frequently in micropropagated 'Cavendish' bananas (Israeli *et al.*, 1991; Smith and Drew, 1990b; Stover, 1988). Vuylsteke *et al.* (1991, 1996) never observed dwarfism or gigantism among micropropagated plantains, although there have been reports of their occurrence in plantain elsewhere (Sandoval *et al.*, 1991).

Table 3. Yield of micropropagated plants of plantain (*Musa* spp., AAB group) and its somaclonal variants

True-to-type cultivar	Bunch mass (kg)	Bunch mass (kg) of somaclonal variants				Reference
		'French'	'Monganga'	'Distorted laminas'	'Drooping leaves'	
Horn	9.2	18.1	–	–	–	Ramcharan *et al.*, 1987
Dwarf Horn	11.7	26.4	–	–	–	Ramcharan *et al.*, 1987
Agbagba	11.3	14.4*	3.2*	3.8*	2.7*	Vuylsteke *et al.*, 1996

*Significantly different ($p \leq 0.05$) from the true-to-type clone using LSD for single pair comparison within row.

Somaclonal Variation in Banana and Plantain Improvement

The use of somaclonal variation in *Musa* improvement can only be realistically assessed by improving our understanding of the nature and extent of somaclonal variation. In the past, these characteristics were not clear and therefore the potential usefulness of somaclonal variation in improvement was overestimated. Some of the advantages of somaclonal variation are that:

- novel, stable variants can arise,
- variation frequencies can be high,
- some useful changes in agronomic traits can arise.

Eventually, new cultivars may be selected. However, there are several disadvantages with somaclonal variation for the purpose of genetic improvement:

- variation events are uncontrollable and unpredictable,
- most variation is not novel and of no apparent use,
- the nature and frequency of variation is genotype-dependent,
- some changes are unstable or non-heritable.

Eventually, the value of somaclonal variation in breeding rests in the ability to recover at high frequency new genetic variants with desirable characteristics. Hence, it would be useful to devise methods to efficiently identify rare and useful somaclonal variants among large populations of useless variants (Evans and Sharp, 1986; Karp and Bright, 1985; Larkin and Scowcroft, 1981).

The banana is especially interesting for investigating somaclonal variation as a tool in crop improvement, because of its vegetative propagation and the relative difficulty of breeding the crop by conventional means. But, unlike in similar crops such as potato (Kumar, 1994), *in-vitro* regeneration techniques in banana are not easy or diverse. Only shoot-tip culture is widely and routinely used for germplasm handling, while callus, cell suspension, protoplast and anther culture techniques are as yet tedious and of limited applicability due to their strong genotypic dependency. Hence, only somaclonal variants obtained from shoot-tip culture have been evaluated to a reasonable extent.

Somaclonal variation from micropropagated banana and plantain should not be overestimated as a source of novel variability for use in genetic improvement (Vuylsteke *et al.*, 1991, 1996). The range of somaclonal variants recovered through shoot-tip culture is narrow and mostly mimics naturally occurring variation. The agronomic performance of somaclonal variants is generally inferior to that of the original clone from which they were derived. The overall poor yield of off-types is actually limiting the expansion in use of *in-vitro* plants, which otherwise have many advantages and are particularly useful when a new genotype is being introduced. This has been demonstrated with the commercially important dessert banana cultivars of the 'Cavendish' subgroup, in which most somaclonal variants are agronomically inferior to the original clone (Daniells and Smith, 1993; Hwang and Ko, 1987; Smith and Drew, 1990b; Stover, 1987). Some off-types may have beneficial traits, such as the higher yield of the 'French reversion'

variant in plantain, the short stature of dwarfs and the lower *Fusarium* wilt incidence in variants of 'Cavendish' banana, but these changes are always associated with problems in fruit size and quality (Hwang *et al.*, 1993; Stover, 1987; Vuylsteke *et al.*, 1996). Also, the generation of somaclonal variants is a random process and screening at the whole plant level for somaclonal variants with disease resistance requires considerable space, time and labour. Large-scale surveys of somaclonal variants of banana and plantain did not indicate any increase in resistance to the sigatoka leaf spot diseases (Stover, 1987; Vuylsteke *et al.*, 1991), although a recent report from Venezuela may prove the contrary (Trujillo and Garcia, 1996). The field resistance to *Fusarium* wilt of somaclonal variants recovered in Taiwan (Hwang and Ko, 1987; Hwang *et al.*, 1993) remains to be confirmed and tested in other locations. In addition, recent advances in the genetic improvement of *Musa* by conventional and gene transfer methods (May *et al.*, 1995; Rowe and Rosales, 1996; Sagi *et al.*, 1995; Vuylsteke *et al.*, 1997) suggest that such targeted approaches hold much more promise than somaclonal variation *per se*.

However, because the somaclonal variation derived through shoot micropropagation may only represent a fraction of the variation that could be generated through regeneration from cell and protoplast cultures from different explants, the full potential of somaclonal variation for plantain and banana improvement remains to be explored. Somaclonal variation from cell cultures has been advocated as a powerful option for plant improvement, particularly if it can be combined with *in-vitro* selection for desirable mutations at the cellular level (Evans and Sharp, 1986; Larkin and Scowcroft, 1981). In *Musa*, *in-vitro* selection has been evaluated on shoot-tip cultures for *Fusarium* wilt resistance using fusaric acid, fungal crude filtrates, or conidial suspensions of the pathogen. However, there was no linkage between *in-vitro* and *in-vivo* behaviour, suggesting that *in-vitro* selection using crude filtrate or phytotoxin in a screening programme for *Fusarium* wilt resistance is not feasible (Morpurgo *et al.*, 1994). Conversely, Matsumoto *et al.* (1995) reported the successful use of *in-vitro* selection by fusaric acid on chemical mutagen-induced variants of banana with *Fusarium* wilt tolerance. These contradictory results suggest that more work should be done in this area, particularly as cell suspension culture becomes more readily applicable in *Musa* (Dhed'a *et al.*, 1991; Novak *et al.*, 1989).

Somaclonal variation may also be used as a secondary tool for the genetic improvement of *Musa* (Ortiz and Vuylsteke, 1996). Vuylsteke *et al.* (1995) used somaclonal variation to initiate breeding in the sterile 'False Horn' plantain pool. Sterility is the major limitation in 'False Horn' plantain hybrid production (Vuylsteke *et al.*, 1993b). However, a 'French' somaclonal variant of the 'False Horn' plantain cv. 'Agbagba' showing increased female fertility (Vuylsteke *et al.*, 1991, 1993b) made this plantain pool accessible to conventional breeding schemes. 'PITA-9' is a selected tetraploid hybrid derived from the female-fertile 'French' somaclonal variant of this 'False Horn' plantain. It is resistant to black sigatoka, produces high yields and big fruit in the humid forest zone of Nigeria and Cameroon, and has improved agronomic traits relative to the plantain

landraces. It also has good post-harvest qualities, with fruit quality and shelf-life characteristics similar to those of the 'False Horn' and 'French' plantains (Vuylsteke *et al.*, 1995). A full-sib black-sigatoka-resistant diploid hybrid (TMP2x 1297-3) was also obtained from crosses of the 'French' somaclonal variant (Vuylsteke and Ortiz, 1995). Increased fertility levels allow using 'PITA-9' as a parent for further ploidy manipulations via $4x \times 2x$ crosses and for population improvement within the 'False Horn' plantains. 'PITA-9' has been crossed with its diploid full-sib 'TMP2x 1297-3' with the objective of applying directional selection in the segregating population to obtain progenies with large fruit of acceptable quality. This example demonstrates that the potentials of somaclonal variation as a secondary source of variability could be further explored for *Musa* germplasm enhancement.

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13. Somaclonal Variation in Solanaceous Medicinal Plants

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Introduction

A wide range of pharmaceutical secondary metabolites, derived from several higher plants, have been used to benefit mankind for many thousands of years. Many of these compounds have been known for years, and are still being used as the drugs of choice for specific therapy, e.g. *Digitalis* cardiac glycosides such as digoxin (Parr, 1989). Furthermore, new compounds are being isolated from the plant kingdom, and are actively screened. The chemical synthesis of most of the plant-derived compounds is possible; however, it is usually complicated, requires several steps in the biosynthetic pathway, and is thus uneconomical. Plant cell cultures offer an alternative approach to produce valuable plant compounds and they can also be used in plant breeding (Verpoorte *et al.*, 1993). Plant tissue culture-derived variation among regenerated plants, or somaclonal variation, has been extensively studied, especially in crop plants such as sugarcane, tobacco, rice, maize and barley (Larkin and Scowcroft, 1981; Peschke and Phillips, 1992). However, surprisingly little information is available on the applications of somaclonal variation in medicinal plants and their cell cultures.

This chapter is largely based on experiments done in our laboratory with the Egyptian henbane, *Hyoscyamus muticus* L. We have used it to study somaclonal variation to develop new breeding lines, select high-producing cell cultures, and to understand the genetic basis of somaclonal variation. The data from our work and other investigators suggest that somaclonal variation can become a powerful breeding tool for the improvement of medicinal plants and their cell and tissue cultures.

Plant Secondary Metabolites

Natural pharmaceutical compounds can be derived from micro-organisms, animals or plants. Most of these compounds are classified as secondary metabolites or secondary products and they originate from primary precursors. Secondary compounds have low molecular weight, and unique or complex structures. They are synthesized in certain types of cells of specific plant species at a particular development stage, and are usually stored either in vacuoles or in other differentiated tissues, e.g. glandular hairs. Thus the optimal storage of compounds is dependent on the age of the plant, sometimes requiring several years before plants can be harvested. Secondary compounds are not believed to have any essential role in the basic life process of the plant. However, they are ecologically important in the

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interaction of the organism with the environment. For instance, they protect plants from attack by micro-organisms and pests, and they also attract insects.

Plant-derived drugs and intermediates account for approximately US$9–11 billion annually in the US market alone (Principe, 1989). The present value of current plant-based pharmaceuticals is estimated at US$400–600 billion up to the year 2000 in OECD countries. Some compounds have an extremely high price, e.g. vincristine (from Catharanthus roseus) which is used as an anticancer agent. Others, such as the antitussive drug codeine, are less expensive with a large market volume. Because of their complicated chemical synthesis, many plant-based products are currently isolated from naturally growing or cultivated plants, or their specific plant organs (Oksman-Caldentey and Hiltunen, 1996). In some cases, there is also a risk of over-collecting endangered species (e.g. *Taxus brevifolia* for production of taxol); therefore, it would be desirable to preserve these species by using production methods based on biotechnology (Verpoorte *et al.*, 1993).

Medicinal Plants from Solanaceae

The most important group of chemicals, produced by the Solanaceous medicinal plants, are: tropane alkaloids atropine, *l*-hyoscyamine and scopolamine. They are anticholinergic agents that affect the parasympathetic nervous system and exhibit a wide range of pharmacological activity. In medicine, they are used to treat Parkinson's disease, to dilate the pupils and increase the heart rate, to counteract organophosphate poisoning that causes smooth muscle relaxation, and to reduce secretion such as sweat and gastric acid. Scopolamine has a stronger effect on the central nervous system than hyoscyamine, but causes fewer undesirable side effects, and is used for the treatment of motion sickness and the production of derivative drugs for gastric disorders. These alkaloids are produced by several plants belonging to *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus*, *Mandragora* and *Scopolia* genera.

Capsicum annuum L. and *C. frutescens* L. are typically used as spices, and produce capsaicin. Capsaicin is a pungent substance which is used as a rubefacient in liniments for the symptomatic treatment of rheumatic pains. It increases the blood circulation in the skin.

Nowadays, *Nicotiana tabacum* L. and its related species, e.g. *N. rustica* L., are not considered as valuable medicinal plants, although their alkaloids have very strong physiological properties. The main alkaloid is nicotine, a pyridine alkaloid, which is an oil. Nicotine is a strong poison, and has been used as an insecticide in gardening. James and Nordberg (1995) reported the possibility of using nicotine in the treatment of Alzheimer's disease. Moreover, tobacco as a stimulant has been known for centuries. The production of nicotine and its derivatives nornicotine, lobeline and anabasine has been widely studied including their production in plants and cell cultures as model systems (Wernicke and Thomas, 1980; Furze *et al.*, 1987; Fecker *et al.*, 1993).

Solanum tuberosum L., potato, contains the glycoalkaloid solasodine, which is an alkaloidal analogue of diosgenin. Unripe fruits of other *Solanum* species, e.g.

S. laciniatum Aiton, *S. aviculare* G. Forster and *S. khasianum* C.B. Clarke, also contain solasodine, and they serve as a raw material for the partial synthesis of corticosteroids and contraceptives.

Plant Cell and Tissue Cultures: Alternative Source for Plant-derived Drugs

The production of pharmaceutical secondary metabolites using plant cells has been the subject of intensive research in recent years (see Parr, 1989; Verpoorte *et al.*, 1993; DiCosmo and Misawa, 1995; Oksman-Caldentey and Hiltunen, 1996). A number of substances have been isolated from plant cell cultures including several medicinally important compounds. The expectations were high ever since the first commercial product – shikonin – with the plant cell culture technique of *Lithospermum erythrorhizon* Sieb. & Zucc. was commercially produced (Fujita and Tabata, 1987). In spite of huge efforts, only a few products have reached the commercial stage since then. The main reason has been either low product yield or instability in production.

Callus and cell suspension cultures that represent the undifferentiated cells are grown on solid and liquid media, respectively. Both types of cultures often need auxins and/or cytokinins for their growth, as well as for the accumulation of secondary products. An alternative method of producing biologically active compounds involves the use of differentiated shoot and root cultures because their behaviour is more predictable than that of undifferentiated cultures (Parr, 1989). Differentiation is achieved with an external hormone balance. Many studies have recently shown that transformed root cultures offer better possibilities of producing secondary metabolites than the intact plant or any other method described earlier (Flores *et al.*, 1987; Rhodes *et al.*, 1990; Oksman-Caldentey and Hiltunen, 1996).

Cell suspension cultures can also be grown on a large-scale in bioreactors (Scragg, 1992). However, plant cells grow more slowly than micro-organisms and are more sensitive to shear stress; therefore, special bioreactors are designed for cell suspension growth. Although large-scale cultivation of differentiated cultures is also possible, it is much more difficult than that of cell suspension cultures (Wilson *et al.*, 1990).

Somaclonal Variation and its Genetic Nature

The genetic variability in tissue culture-derived plants has been documented in several studies, and was termed 'somaclonal variation' by Larkin and Scowcroft (1981). The major factors that appear to affect somaclonal variation are: genetic background (genotype), explant source (variation pre-existing in the tissue), characteristics of the initiated growth form (meristem versus callus), medium composition (e.g. effect of 2,4-dichlorophenoxyacetic acid) and culture age (Karp, 1991; Jain *et al.*, 1998a).

Somaclonal variation can arise either from genetic or epigenetic changes (Larkin and Scowcroft, 1981; Meins, 1983; Karp, 1995). Firstly, chromosomal

changes, including changes in chromosome number (polyploidy and aneuploidy) may induce genetic variation, although, it is likely that many gross karyotypic changes will be selected against during plant regeneration, especially in diploids (Sree Ramulu *et al.*, 1985; van Altvorst *et al.*, 1992; Kawata *et al.*, 1992; Gupta, 1998). Cryptic chromosomal rearrangements, such as chromosome breakage, translocation, deletion and inversion, are to a large extent responsible for the genetic variation in plant cell cultures. Secondly, translocation, deletion and inversion could also occur at the gene level, and these effects are usually minor compared to the chromosomal changes. Cryptic changes can result in the loss of genetic material or the function, but it can also express genes which have hitherto been silent. Transposable genetic elements of DNA, which are able to move from one locus to another in the genome, play an equal role in somaclonal variation (Peschke *et al.*, 1987). It seems likely that somatic gene rearrangements also occur in higher plants and generate new germlines. Specific genes of higher organisms can amplify themselves during differentiation or in response to environmental pressures (epigenetic changes), which may partly cause somaclonal variation (Meins, 1983). Some of the somaclonal variation may arise by an increase in the frequency of somatic crossing-over. Somatic cell – sister chromatid exchanges can sometimes lead to deletion and duplication of genetic material. Finally, so-called cryptic virus elimination consists of a wide range of reactions that could result in somaclones that react differently to the fungus infection (Kassanis *et al.*, 1977).

Improvement of Secondary Metabolite Production using Somaclonal Variation

Despite limited successful examples, the production of secondary metabolites in unorganized plant cell cultures has usually remained low. Besides low yield, the culture instability causes additional problems (e.g. Oksman-Caldentey and Strauss, 1986). This might be due to the inhibition of enzymes in the biosynthetic pathway; and partial expression of the key-enzymes or other non-enzymatic factors, such as lack of precursors or accumulation capacity of the metabolite. Also, the catabolism of the product may result in minimal production capacity. Several trials have been carried out to improve the production (Table 1).

Some studies have been conducted to optimize the growth conditions as well as the culture medium (Oksman-Caldentey *et al.*, 1994; Hilton and Wilson, 1995; Nuutila *et al.*, 1991; Toivonen *et al.*, 1991; Hilton and Rhodes, 1994) with limited success. The use of organized cultures has often improved the productivity of secondary metabolites (Oksman-Caldentey and Hiltunen, 1996). However, the conventional root cultures tend to grow slowly (Parr, 1989). On the other hand, biotic and abiotic elicitors have stimulated secondary product formation both in undifferentiated and differentiated cultures (Eilert, 1987; Arroo *et al.*, 1995; Sevón *et al.*, 1992). However, the main problem in applying any one of the methods [presented in Table 1] is the lack of basic knowledge on the biosynthetic pathways,

Table 1. Possibilities to increase secondary product formation in plant cell and tissue cultures

Optimization of the growth medium
Optimization of external growth conditions
Use of various elicitors
Use of differentiated cultures
Biotransformation
Somaclonal variation
Use of genetic engineering

and the mechanisms for the production of secondary metabolites, especially in the regulation of biosynthesis. No real progress has been made in identifying the biosynthetic enzymes before the use of plant cell cultures. Since then, over 80 new enzymes that catalyse biosynthetic steps of various alkaloids have been discovered. Some of them are also either fully or partially characterized (Kutchan, 1995). In the future, genetic engineering will certainly help us to improve the production of valuable secondary products in plant cell and tissue cultures (Oksman-Caldentey and Hiltunen, 1996).

Somaclonal variation has been widely used as a breeding tool and to select important traits for crop improvement (Larkin and Scowcroft, 1981). Plant cell cultures are heterogeneous and it is, therefore, possible to select cells with a desirable trait, e.g. resistance against abiotic and biotic stresses, high-yielding crop plants, modification of ornamental plants (Jain *et al.*, 1997; 1998b), etc. Moreover, it can also be applied to the medicinal plant development and to screen high secondary metabolite-producing cell lines or clones (Furze *et al.*, 1987; Oksman-Caldentey, 1987). The ideal way to study somaclonal variation is to evaluate variation among plants regenerated from tissues or cell clones. Protoplasts are also an ideal system for this type of study (Orton, 1983; Sevón *et al.*, 1995; 1997b).

Somaclonal Variation in Undifferentiated Cell Culture Clones

Several investigators have reported wide differences in yields of phytochemicals within clones, obtained from plant cell cultures (Oksman-Caldentey *et al.*, 1987a; Sevón *et al.*, 1997a). The techniques used in the selection and screening of plant cell cultures for improved product synthesis are based on cell cloning methods, such as pigmentation, fluorescence assays, product analysis using rapid radioimmunoassays, etc. The selection of highly productive somaclonal cell lines by cell cultures has been a well-known strategy for the production of important pharmaceutical secondary metabolites (see Zenk *et al.*, 1977; Mano *et al.*, 1989). However, few studies have been conducted on variability in plant cell cultures to distinguish the variation in secondary metabolite yield, arising either from heterozygosity of the original explant or from single cells (e.g. from protoplasts within the culture). These studies show that clones which give rise to subclones displaying a wide range of yields (Deus-Neuman and Zenk, 1984; Oksman-Caldentey *et al.*, 1987a; Sevón *et al.*, 1997a). Some more detailed examples are given below.

Systematic studies carried out on two cultivars of *Hyoscyamus muticus* revealed considerable somaclonal variation in cell clones derived from mesophyll protoplasts (Oksman-Caldentey and Strauss, 1986; Oksman-Caldentey *et al.*, 1987a), especially in two main tropane alkaloids as well as in the growth rate and morphology of the clones. Cell clones obtained from haploid plants produced 2–3 times more alkaloids than those derived from diploid ones. Also, the variability among haploid clones (115%) was higher than among the diploid population (56%) (Oksman-Caldentey and Strauss, 1986). Somaclonal variation in haploid clones, which are either true haploid or homozygous diploid, cannot be due to heterozygosity of genetic factors affecting alkaloid content. The frequency distribution of the scopolamine content was weakly bimodal (Fig. 1). These results indicated that the clonal series contains a minor subpopulation with relatively high scopolamine content and possibly having a different genetic composition (Oksman-Caldentey and Strauss, 1986). Thus, clones producing 300 times more scopolamine than the others could be selected from the somaclone population (Oksman-Caldentey, 1987). This type of large variation exceeds by far the variation obtained from other feasible sources such as extraction procedures, dilutions, assays or the possible heterogeneity of the clones. Even differences in the growth pattern cannot explain this large variation.

The spontaneous appearance of variation amongst subclones of a given parental cell line is a relatively common phenomenon. Zenk (1978) reviewed that the callus subclones of *Solanum laciniatum* possessed variation in their ability to produce alkaloids, by examining 143 subclones. The production of steroidal alka-

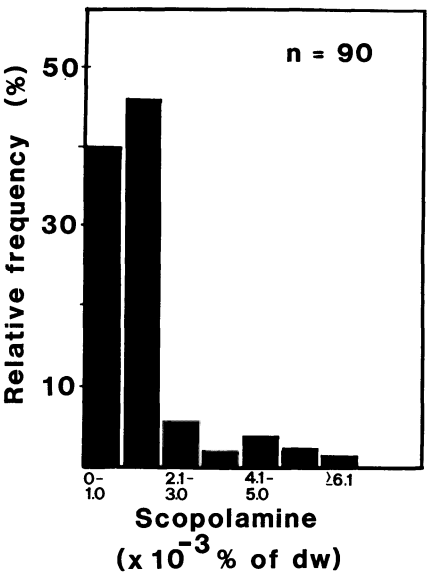

Figure 1. Frequency distribution of scopolamine content in a population of cell culture clones derived from a haploid *Hyoscyamus muticus* plant (Oksman-Caldentey and Strauss, 1986)

loid solasonine varied from 0% to 3% on a dry-weight basis. Similar results were also obtained in nicotine production from *Nicotiana rustica* and *N. tabacum* cultures (Tabata and Hiraoka, 1976; Tabata *et al.*, 1978). Plants regenerated from variants with high nicotine content did not contain more nicotine than the control ones. The fresh cultures initiated from these regenerated plants produced about 6-fold more nicotine than the control cultures. This type of variation may arise due to genetic rearrangements and expression of multiple genes that are responsible for the production of secondary metabolites. On the other hand somatic hybridization between N. rustica and N. tabacum resulted in increased contents of nicotine in the progenies (Pandeya *et al.*, 1986).

A suspension culture of *Hyoscyamus niger* L. was established and sieved to obtain a fine suspension with small cell aggregates. Colonies were later formed from small aggregates, and they showed variation in their morphology. Thirteen out of 71 colonies formed root primordia. Roots were separated and well-growing root cultures produced scopolamine as the main alkaloid contrary to cell suspension cultures in which hyoscyamine was the pre-dominant alkaloid (Hashimoto and Yamada, 1983). Yamada and Hashimoto (1982) selected hyoscyamine-rich callus culture based on somaclonal variation. Line H15 was selected out of 70 newly induced calli, and it produced hyoscyamine at 0.02% level of dry weight. However, when cell suspension cultures were initiated from this line, hyoscyamine production decreased. This is a very common phenomenon among undifferentiated cell cultures. Moreover, cell line H15 was not initiated from a single protoplast. Usually, this approach increases variation in the following passages and most probably decreases secondary product formation.

Immobilization can also have an influence on cell morphology, and induce cells to respond differently to changing conditions due to their own genetic differences. *Solanum aviculare* cells were immobilized within calcium alginate gel beads and most of the immobilized cells showed an ellipsoidal or spherical morphology with brown pigmentation, while others showed an elongated morphology without pigmentation. Both cell types differed in their abilities to release secondary products into the medium (Roisin *et al.*, 1997).

Somaclonal Variation in Differentiated Cell Culture Clones

Differentiated cell and tissue cultures, such as root and shoot cultures or embryogenic cultures, are known to be more stable during *in vitro* cultivation than undifferentiated cell suspension or callus cultures (Verpoorte *et al.*, 1993). Furthermore, their secondary metabolite content is usually much higher. The secondary metabolite production, however, is highly linked to cell differentiation. Alkaloid production decreases clearly when roots are induced to form callus, and reappears when roots are allowed to redifferentiate (Flores *et al.*, 1987; Sevón *et al.*, 1997a). Moreover, differentiated cultures cannot be influenced very easily by external factors like the use of elicitors (Sevón *et al.*, 1992; Oksman-Caldentey *et al.*, 1994).

Transformed hairy root cultures

During recent years intensive studies have focused on secondary metabolite production in genetically modified transformed root cultures, also termed hairy root cultures (see Oksman-Caldentey and Hiltunen, 1996). The hairy root formation is a result of the transfer of *Agrobacterium rhizogenes* genes into the plant genome. These transformed roots can be excised from the wounded plant and then can be cultured under *in vitro* condition. Cultured hairy roots produce secondary metabolites characteristic of the mother plant, and production by this approach has been shown to be stable (Maldonado-Mendoza *et al.*, 1993; Sevón *et al.*, 1997a). Chilton *et al.* (1982) suggested that each individual hairy root obtained after the transformation is derived from a single cell. Generally, a hairy root is regarded as a clone, although there is some speculation about its true nature (Yukimune *et al.*, 1994; Sevón *et al.*, 1995).

Tropane alkaloids that are scarcely synthesized in undifferentiated cells are produced at a relatively high level in cultured roots. This suggests that alkaloid production is associated with the organogenesis of roots (Endo and Yamada, 1985). Mano *et al.* (1986) established 125 hairy root clones of *Scopolia japonica* Maxim. They selected 29 rapidly growing clones and further studied their properties. The clones displayed various phenotypes characterized by growth rate, opine production and tropane alkaloid formation. Both agropine and mannopine were found in 14 clones, and mannopine in nine clones without any traces of agropine. Six clones did not produce any of the opines. The growth indices varied from 5 to 52. The contents of scopolamine and hyoscyamine varied from clone to clone. The lowest scopolamine production was only 5% of the highest one. By screening all these clones, they could establish one clone of each high producer of scopolamine and hyoscyamine, respectively, and showed high stability with the subsequent subcultures on the fresh medium (Mano *et al.*, 1986). The various phenotypes of hairy root clones of *S. japonica* may be attributed to differences in the length and copy number of the T-DNAs integrated into the plant genome. In addition, the expression of integrated T-DNA and/or their related genes is able to affect the growth and the synthesis of opines and alkaloids.

A few years later, Mano *et al.* (1989) obtained 45 hairy root clones of *Duboisia leichhardii* F.v.M. from individual root meristems. They also found a considerable variation in growth rate, alkaloid content and productivity of these clones. Based on this variation, they selected high-producing hairy root clones. However, hairy roots also possessed a certain amount of heterogeneity even though they are derived from a single root tip. The repeated selection is applicable to hairy root cultures for obtaining high scopolamine-producing lines (Yukimune *et al.*, 1994). Although, the variation is great among different root clones, the overall production in each root line is stable over years in *Datura stramonium* L. or *H. muticus* hairy root clones (Maldonado-Mendoza *et al.*, 1993; Sevón *et al.*, 1997a).

Long-term studies were performed in our laboratory to investigate the homogeneity of hairy roots. We established hairy root clone populations derived from the same starting material under similar experimental conditions. Large variation was observed in hyoscyamine content among individual root clones obtained from the same transformation experiment. For example, the hyoscyamine production of

100 hairy root clones varied from 0.03% to 0.59% of dry weight (Fig. 2). The frequency distribution was not normal but positively skewed, and resembled the distribution of an undifferentiated cell culture clone population (Fig. 1). This encouraged us to investigate further to select hairy root clones with increased hyoscyamine levels by screening protoplast-derived hairy roots.

Protoplast-derived hairy root clones

Protoplasts have mostly been isolated from leaf mesophyll cells or cultured cells (Wernicke and Thomas, 1980; Kitamura *et al.*, 1989). There are only a few reports of protoplasts having been isolated from roots (Cocking, 1985; Schaerer and Pilet, 1991; Sevón *et al.*, 1995). The direct isolation of protoplasts from hairy roots is not a simple procedure and, therefore, protoplasts have earlier been isolated from cell suspension cultures derived from hairy roots (Furze *et al.*, 1987). We succeeded in isolating the protoplasts directly from the hairy root clone LBA-F (class 7 in Fig. 2) that represented one of the clones containing the highest hyoscyamine content (0.57%) in the whole population (Sevón *et al.*, 1995, 1997a). Altogether, 225 clones regenerated roots, and showed marked differences in their growth rate, morphology and tropane alkaloid contents. The frequency distribution of hyoscyamine production showed less bimodality (Fig. 3) than the original clone population (Fig. 2). Moreover, several high hyoscyamine-producing clones could be selected (Sevón *et al.*, 1997a). This study concluded that the initial hairy root clone, from which the protoplasts were isolated, consisted of heterogeneous cells that caused the variation among the protoplast-derived clones, and could not be considered as a 'pure' clone. Yukimune *et al.* (1994) established a high-producing hairy root clone of *D. myoporoides* R.Br. by repeated selection. Although the selection was not based on protoplasts,

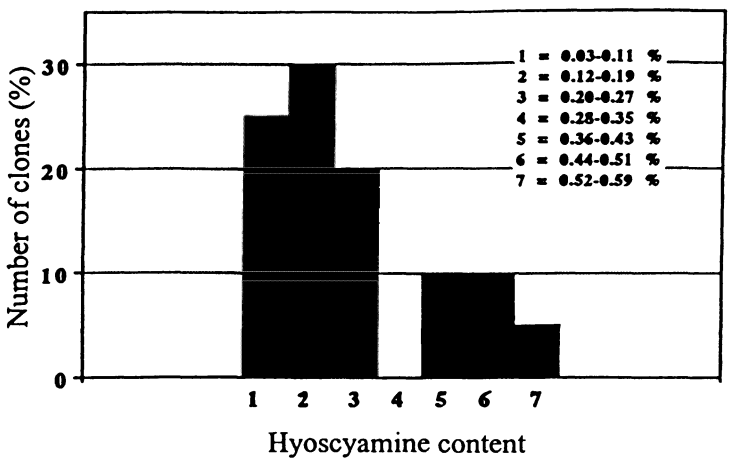

Figure 2. Frequency distribution of the hyoscyamine content between individual root clones (n = 100) obtained from the leaves of the same plant (*Hyoscyamus muticus*) inoculated with *Agrobacterium rhizogenes* strain LBA9402 (Sevón *et al.*, 1997a)

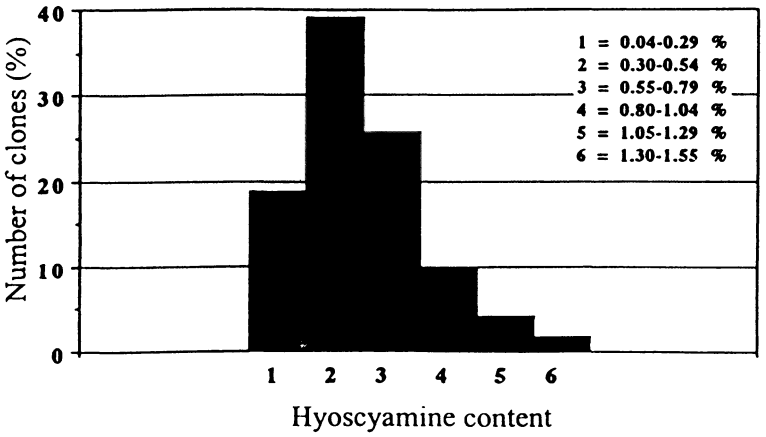

Figure 3. Frequency distribution of the hyoscyamine content in protoplast-derived hairy root clones (n = 171) of *Hyoscyamus muticus* (Sevón *et al.*, 1997a)

they also concluded that the high scopolamine-producing hairy root clone was the result of the removal of heterogeneous cells during selection.

The difficulties faced with root-protoplast isolation have led to the use of other tissues such as undifferentiated tissue. However, the undifferentiated state is known to increase somaclonal variation (Larkin and Scowcroft, 1981). This phenomenon was clearly demonstrated by Furze *et al.* (1987), who established a suspension culture from hairy roots of *N. rustica* by phytohormone treatment. Protoplasts were regenerated into hairy roots again, and the clones showed a very high degree of somaclonal variation in morphology, nicotine accumulation, growth rate and the copy number of T-DNAs. Similarly, Sevón *et al.* (1997a) isolated protoplasts directly from the roots and observed a high rate of variation. However, the undifferentiated state in the latter case was very short, and most probably the cause of variation is the initial root clone itself.

Photoautotrophic hairy root cultures

Somaclonal variation has enabled the discovery of photoautotrophic root cultures in some cases. Maldonado-Mendoza and Loyola-Vargas (1995) established two photosynthetic hairy root lines of *D. stramonium*. The detailed characterization revealed that these two lines had diminished growth rate when compared with heterotrophic cultures; increased chlorophyll content, a net oxygen evolution and CO_2 fixation, and also showed *de novo* synthesis of the ribulose 1,5-biphosphate carboxylase enzyme. There was a clear correlation between development of photosynthesis and increase of scopolamine synthesis (Maldonado-Mendoza and Loyola-Vargas, 1995).

Effect of Mutagenization

The concept of selecting simple biochemical mutants among large populations of cultured plant cells that can afterwards undergo regeneration into whole plants

bearing the selected phenomenon has been for a long time the interest of plant geneticists and breeders. This concept could not be materialized effectively, mainly due to the lack of suitable cell cultures and/or plant regeneration system. *In vitro* selection is an efficient method for obtaining plants with desirable traits by having a selective agent in the culture medium. This approach is potentially more efficient in plant improvement than selection at the whole plant level. However, the major limiting factor remains the selection of plants expressing recessive alleles, genes having minor effects on plant phenotype and secondary metabolite production, or influence on gene expression by growth environment (Pauls, 1995).

N-methyl-*N'*-nitro-nitrosoguanidine (MNNG) and 5-bromodeoxyuridine (BUdR) are well-known alkylating agents for inducing mutations in bacteria and plant cells. Also, physical mutagens such as X-rays, UV light, and gamma rays are commonly used for mutagenesis in plant cells (Kartusch and Mittendorfer, 1990). Oksman-Caldentey and Strauss (1986) failed to obtain any scopolamine over-producing clone among 2600 protoplast-derived clones of *H. muticus* by using X-rays and UV light, and chemical mutagen MNNG. The incidence of somaclonal variation was more among the original clones, but clearly reduced in the mutagenized populations. Most probably the population was too small since Gebhardt *et al.* (1981) analysed 29 000 clones and found only 12 biochemical mutants among the whole population (Table 2).

Somaclonal variation has been used to select cell lines resistant against certain mutagens. Such resistance can be achieved, in principle, either by changing the uptake of the chemical or its degradation into an inactive form, or by alterating its

Table 2. Isolation of auxotrophic and temperature-sensitive mutants from mesophyll protoplasts of *Hyoscyamus muticus*

	MNNG dose ($mg.l^{-1}$ 0.5 h^{-1})				
	0	5	10	20	Total
Percentage survival relative to zero dose	100	60	23	8	
A Preselection step	5001	9047	10 140	4684	28 872
B Test 1	138	584	554	431	1704
Test 2	60	315	310	257	942
Test 3	39	225	185	150	599
C (i) Auxotrophic					
amino acids	1	1	3	2	7
vitamins	0	0	2	1	3
bases	0	0	0	0	0
(ii) Temperature-sensitive	0	0	0	2	2
(iii) Total variants	1	1	5	5	
D Number of variants per 10 000 clones	2.0	1.1	4.9	10.7	

* according to Gebhardt *et al.* 1981. The number of clones (**A**) after mutagenization, (**B**) subsequently entering the isolation procedures, (**C**) having a stable variant phenotype

site of action. Ranch *et al.* (1983) isolated 79 cell lines resistant to 5-methyltryptophan (5-MT) from haploid *Datura innoxia* Mill. cell cultures by plating suspensions on a solid medium containing a growth-inhibitory concentration of 5-MT. Mutagen treatment increased the frequency of resistance, and 11 variants had an altered anthranilate synthase, and were less sensitive to feedback inhibition by trypthophan. Five of these variants and the plants selected from these cells contained elevated levels of free tryptophan. These results clearly show that the amino acid overproducing variants can be selected at the cellular level (Ranch *et al.*, 1983). Soon after the initiation of cellular division, exogenous growth regulator requirement for growth seems to change drastically, and thus the selection should preferably be made soon after the protoplast isolation. *In vitro* cell variants, which form clones different from their progenitor cells, can spontaneously arise during less organized proliferation. If these cell clones are well adapted to the prevailing environmental conditions, they will proliferate faster.

Robins *et al.* (1987) reported that nicotinic acid can be used as a selective agent to isolate high nicotine-producing lines of *N. rustica* hairy root cultures. However, the selected root clones also had a higher ability to detoxify nicotinic acid to nicotine and anabasine. Amino acid analogues have also been used for establishing hairy root lines producing a high yield of hyoscyamine. Cell suspension cultures were developed from hairy roots of *H. muticus* and screened for resistance against *p*-fluorophenylalanine (PFP). PFP-resistant cells were selected and induced to differentiate into PFP-resistant hairy roots with elevated levels of hyoscyamine (Fig. 4).

It is now possible to introduce specific genes into plants that regulate the biosynthetic pathway of certain secondary products (Kutchan, 1995). Improvement in the formation of the desirable products has been possible in a few cases (Yun *et al.*, 1992; Hashimoto *et al.*, 1993). Hamill *et al.* (1990) could show that over-expression of ornithine decarboxylase gene (ODC) from the yeast *Saccharomyces cerevisiae* by the CaMV35S^{++} promoter (with the duplicated enhancer sequence) significantly enhanced nicotine accumulation in transformed hairy root cultures of *Nicotiana rustica.* The mean nicotine increase was 2-fold although somaclonal variation was seen between various clones. Moreover, ODC gene and consequently enhanced enzyme activity promoted also the accumulation of free putrescine and free *N*-methylputrescine, whereas there was practically no influence on conjugated putrescine or the amounts of other polyamines such as spermine and spermidine (Hamill *et al.*, 1990). In a similar way, Hashimoto and co-workers (1993) showed enhanced scopolamine production in *Atropa belladonna* hairy roots after expressing the hyoscyamine- 6β-hydroxylase gene (H6H) by CaMV35S promoter. They studied the alkaloid production in seven different transformed root clones and also found variation in the production between these clones (Hashimoto *et al.*, 1993). Several hairy root clones of *N. tabacum*, which carried a bacterial lysine decarboxylase gene, showed an increased cadaverine and anabasine production as compared with the controls. However, great differences were seen among different clones in alkaloid production and NTPII protein (Fecker *et al.*, 1993). Thus, the introduced gene can be expressed differently in various cells and cause changes in secondary metabolite production.

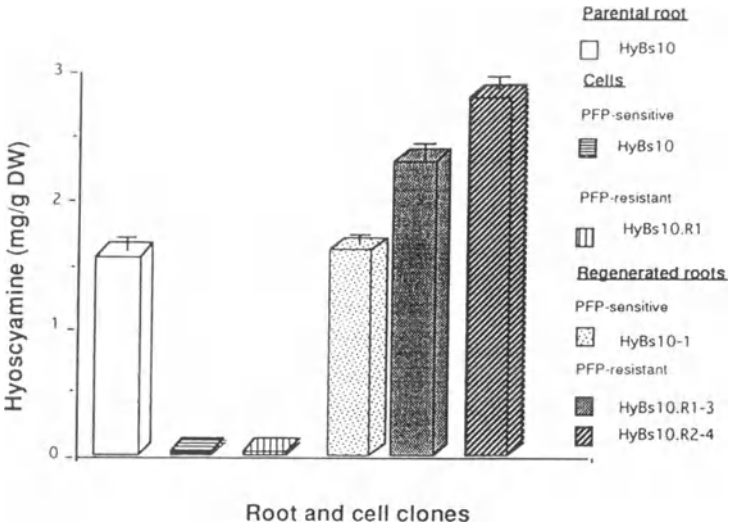

Figure 4. Hyoscyamine contents in the parental hairy root clone (HyBs10), root-derived PFP-sensitive and PFP-resistant cells, and regenerated PFP-sensitive and PFP-resistant hairy roots of *Hyoscyamus muticus* (Medina-Bolívar and Flores, 1995)

Plant Breeding Aspects

Homozygosity is required for plant varieties to ensure their uniformity in the field as well as their constant stability over years. Generally, it takes several years to achieve homozygosity in a field crop by conventional techniques. This time can be shortened by using haploid plants. Moreover, plant cloning can be successfully used to propagate desirable genotypes. Somaclonal variation occurs in both asexually and sexually propagated plants, and self-fertilized species (Larkin and Scowcroft, 1981), e.g in polyploids, such as sugarcane, potato and tobacco, in diploid and even in haploid species including some medicinal plants. In general, the number of genetic changes occurring among tissue culture-regenerated plants is very high as compared to the seed-propagated plant populations (Meins, 1983 Sevón *et al.*, 1997b).

In tobacco, there have been a number of reports on genetic variation indicating that somaclones derived from cell cultures differed significantly among themselves and from the parental line in all the characters examined, including biomass yield and total alkaloid content (Larkin and Scowcroft, 1981).

We performed an extensive study on somaclonal variation of *H. muticus* plants. Scopolamine and hyoscyamine contents in wild-type plants are rather low, on average 0.1% and 0.5% of the dry weight, respectively (Oksman-Caldentey *et al.*, 1987b). We could increase the tropane alkaloid contents when an individual inbred plant selection system was applied. The improved production of the progenies was an inherited attribute resulting from the systematic selection of plants for high-alkaloid contents (Fig. 5). Oksman-Caldentey (1987) demonstrated by breeding experiments that the scopolamine production trait was inherited by the progenies of both parents. Extremely high scopolamine contents (over 4.4% of

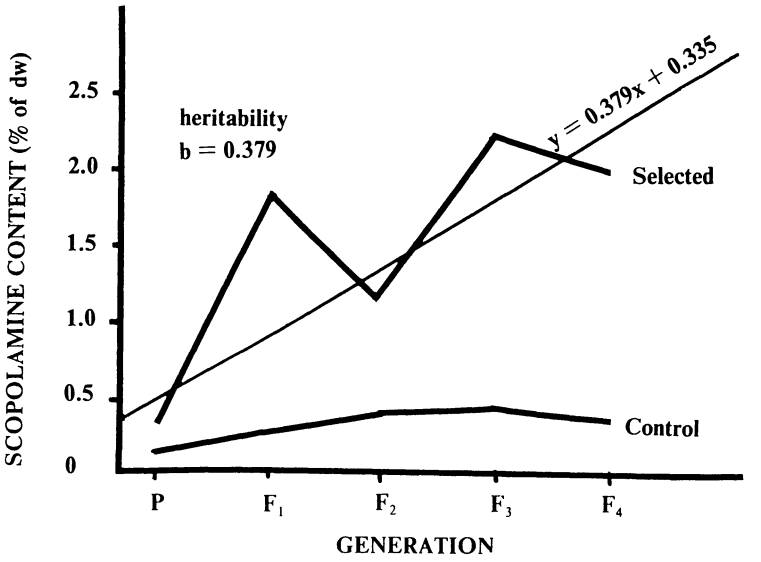

Figure 5. Improvement of scopolamine production of *Hyoscyamus muticus* plants in four successive generations by systematic inbred selection (Oksman-Caldentey, 1987)

dry weight) were detected in F_4 generation at levels so far not reported for any *Hyoscyamus* species or exceeding levels found in *Duboisia*, which is the principal source of commercial scopolamine (Oksman-Caldentey *et al.*, 1987b).

Recently, mature plants have been regenerated directly (Oksman-Caldentey *et al.*, 1991) or via protoplasts from *A. rhizogenes* transformed root cultures of *H. muticus* (Sevón *et al.*, 1995). The regenerated plants showed strong phenotypic clonal differences (Table 3). Moreover, within the same clone, the individual plants differed in morphology and tropane alkaloid production (Table 4). This is the first time that somaclonal variation has been investigated in a statistically significant number of hairy root-derived plants (Sevón *et al.*, 1997b).

Advantages and Disadvantages of Somaclonal Variation

Protoplasts offer an excellent system for producing single cells and studying somaclonal variation. Protoplasts could be used as a means to circumvent the problems associated with the lack of definition of the explants, but the necessary assumption of genetic uniformity of different protoplasts still remains to be established. The totipotency of protoplasts of many plant species has raised the possibility of improving higher plants by genetic engineering. However, this approach relies on screening of a large cell population, e.g. tens of thousands of cell lines. Moreover, repeated selection is sometimes required to ensure that the selected properties are stable. Stable cell lines can be maintained by cryopreservation. This system still needs improvement, since viability after cryopreservation has until now remained too low.

Table 3. Characterization of the regenerated plants of *Hyoscyamus muticus* (Sevón *et al.*, 1997b)

	P2 ($n = 12$)	P12* ($n = 10$)	P219 ($n = 1$)	P224 ($n = 6$)	P241 ($n = 8$)	P277 ($n = 7$)	Control ($n = 10$)
Adaptation to soil	Difficult	Impossible	Extremely difficult	Difficult	Difficult	Very difficult	Easy
Height (cm)†	23.9 ± 6.9	–	9.0	23.2 ± 9.5	23.4 ± 5.7	18.8 ± 2.8	55.0 ± 3.0
Apical dominance	Normal	Reduced	Reduced	Normal	Normal	Reduced	Normal
Root formation	Long, few branches	Highly branched	Very thin	Thin, few branches	Many, highly branched	Thin, few branches	Normal
Tillering	–	+	+	–	–	+	–
Plagiotropic growth of stems	Increased	–	–	Increased	Increased	–	–
Leaf morphology							
Wrinkling	No	No	Increased	No	Highly increased	Increased	No
Size	Narrow	Narrow, small	Wide, small	Narrow, small	Wide	Small, round	Normal
Colour	Green	Light green	Dark green	Green	Green	Dark green	Green
Shape of edges	Smooth	Smooth	Smooth	Smooth	Indented	Indented	Indented
Number of leaves	Normal	Greatly increased	Increased	Normal	Normal	Increased	Normal
Time of flowering	7 weeks	–	10 weeks	7 weeks	7 weeks	11 weeks	4 weeks
Plants setting flowers	12	0	1	6	8	5	10
Flower morphology	Abnormal	–	Abnormal	Abnormal	Abnormal	Abnormal	Normal
Number of stamens	4–6	–	3	5–7	5–15	5–7	5
Seed capsules	Small, few	–	None	Small, few	Normal, few	Small, few	Normal, many
Seed setting	None	–	None	None	None	None	All

*Characterization is based on *in vitro* conditions.

†At flowering time.

Table 4. Changes in scopolamine content during different growth periods in leaves of regenerated and control *Hyoscyamus muticus* plants

	Scopolamine content (mg/g dray weight ± SD) mean + SD					
Growth period	P2 (n = 12)	P219 (n = 1)	P224 (n = 6)	P241 (n = 8)	P227 (n = 7)	Control (n = 10)
I	0.19 ± 0.18	<0.01	0.14 ± 0.18	1.11 ± 0.85	0.01 ± 0.01	1.15 ± 0.82
II	1.48 ± 0.88	0.01	1.13 ± 0.33	2.78 ± 1.48	0.03 ± 0.04	2.61 ± 0.71
III	1.53 ± 0.63	0.01	1.48 ± 0.47	4.61 ± 1.67	0.12 ± 0.15	4.50 ± 1.27
IV	1.21 ± 0.79	0.01	0.87 ± 0.58	3.76 ± 2.49	0.05 ± 0.05	3.81 ± 3.07
V	0.49 ± 0.68	<0.01	1.27 ± 0.93	3.15 ± 3.04	0.05 ± 0.05	0.82 ± 0.41

I: young plants with max. 5 leaves; II: young plants with max. 10 leaves; III: plants before flowering; IV: plants during full flowering; V: plants after flowering. (Sevón *et al.*, 1997b)

There is no complete agreement on the reasons for diversity in alkaloid or other secondary metabolite production among clones. However, the examples mentioned above as well as others from non-medicinal plants support the generality of this phenomenon. Somaclonal variation arises as a result of genetic changes which may alter the gene expression of the cells and the synthesis of secondary metabolites. Genetic changes certainly cause the occasional variability in secondary metabolites, but the expression of many secondary pathways is easily altered by external factors. Furthermore, the responses of the cells to external factors depend on their physiological stage. However, a different level of a particular metabolite is the result of differential and reversible gene expression (Berlin, 1990).

In conclusion, the phenomenon of somaclonal variation combined with targeted metabolic engineering may in the future prove a valuable tool in medicinal plant improvement.

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SECTION 2

14. Induced Mutation in Plant Breeding: Current Status and Future Outlook

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Introduction

Variability is highly essential to allow a breeder to select cultivars of plants which are: (a) more adapted to environmental changes, (b) more efficient in utilizing nutrients, (c) more tolerant to diseases and pests, and (d) improved in yield and quality. There is a continuous change with time, in natural and man-made environments and human population needs. Therefore, our continuous efforts should be directed towards tailoring the new cultivars, that are capable of withstanding continuous changes and fulfilling human food demand. Selection is the driving force of plant breeders to identify within a variable population the best genotypes which respond to the demands of agricultural producers, the agro-industry and consumers.

The more important factors in producing variability in plants are hybridization, recombination and mutation (spontaneous or induced). However, the naturally occurring mutation rate is too low for practical applications. Therefore, physical and chemical mutagens have proven useful for increasing the frequency of mutations and variation. In this regard mutation breeding is considered complementary to the conventional cross method, with the difference that the former usually causes discontinuous variability with unknown limitations. Both approaches are used for re-creation of genetic variation that was lost, either by too rigid selection or by narrow-based germplasm introduction.

Mutagens cause random changes in the nuclear DNA or cytoplasmic organelles, resulting in gene, chromosomal or genomic mutations (e.g. deletions, translocations, duplications, aneuploidy, breakage of linkage association, etc.) and create variability. The limitation of mutation breeding is the unspecificity of the mutated trait. Mutation represents a unique analytical tool to study gene function and to understand the molecular basis of inheritance of plant characteristics. A large mutant germplasm collection of different model plants (*Arabidopsis, Brassica*, etc.) and crop plants (wheat, pea, barley, etc.) is available for genetic and plant transformation studies. In this chapter the aspects of mutation induction, the contribution of induced mutations to plant breeding and the role of mutagenesis in fundamental studies will be briefly reviewed. The major achievements of applied mutagenesis and its perspectives will also be presented and discussed.

S.M. Jain, D.S. Brar, B.S. Ahloowalia (eds.), Somaclonal Variation and Induced Mutations in Crop Improvement, pp. 255–291.

Concept and Significance of Mutations

Concept of Mutation

The term 'mutation' was introduced by de Vries (1901) to designate a sudden change in a character which was heritable and, therefore, transmissible to the progeny. Changes in the genetic material can occur at three different levels:

1. at gene level (gene mutation), changes in the molecular structure of the gene;
2. at chromosomal level, chromosomal structure changes such as transverse breakage of the chromosome and eventual reunion of the broken ends to form new types of chromosomes;
3. at genome level (genomic mutation), such as addition or loss of a single chromosome (aneuploidy) or doubling of a chromosome complement (polyploidy).

Spontaneous Mutations

Mutations can occur without any apparent cause. However, a closer analysis of their origin reveals that intrinsic and/or extrinsic factors of the plant may be responsible for the spontaneous mutations. Important intrinsic factors are the genetic constitution and the physiological conditions of the plants.

Baur (1924) and Stubbe (1934) described spontaneous mutations for quantitative characters in *Anthirrinum majus* when they observed an unexpected high increase in spontaneous genetic variation for many quantitative traits in a stock obtained by diploidization of haploid plants. Thereafter, these mutations were found to be genetically uniform in tobacco (East, 1935) and in maize (Schuler and Sprague, 1956).

Giles (1940, 1941) showed in *Tradescantia* that the genetic imbalance due either to hybridity or polyploidy might be sufficient for the observed frequencies of aberrations. In most hybrids, however, the majority of genetic disturbances resulted from heterozygosity for chromosomal changes, such as inversions and translocations. Another source of genetic variation is inherent to the mitotic mechanisms of unstable chromosomes such as dicentric chromosomes, centric rings and telocentric chromosomes.

Following the initial investigations of Navashin (1933) on ring chromosomes in *Crepis*, the classical studies of McClintock (1938, 1941) on the behaviour of ring and dicentric chromosomes in maize have clarified the behaviour and fate of these unstable chromosomes. In addition to maize, spontaneous occurring rings and dicentrics were found to persist in subsequent cell generations of other plants such as bread wheat (*Triticum aestivum*) (Sears and Camara, 1952), *Narcissum* (Darlington and Wylie, 1953) and *Agropyron scabrum* (Hair, 1953). The transmission of the dicentrics in radiation-induced dicentrics in *durum* wheat (Bozzini, 1961) and in pea (Monti and Saccardo, 1969) is of special interest. Mutator genes are known to control the spontaneous mutation rate in maize (Rhoades, 1941, 1945).

Internal factors such as the physiological conditions and metabolism of the plants are possibly responsible for spontaneous mutations. De Vries (1901) discovered that in aged seeds of *Oenothera* the rate of spontaneous mutations was highly increased. Better seed storage conditions have been found (D'Amato, 1964a) to reduce, but not to eliminate, the occurrence and accumulation of mutations in seeds.

Nutrition, temperature, naturally occurring radiation and chemicals, and very high oxygen pressure are listed among the external factors responsible for spontaneous mutations. A very high incidence of chromosome breakages, up to 17 times more than the control, occurs in *Tradescantia* plants grown in magnesium or calcium deficiency conditions (Steffensen, 1953, 1955).

The action of abnormal temperature, which may occur in particular environments, seems to be of great relevance for naturally occurring mutations. Bruhin (1951) found that in alpine populations of *Crepis capillaris* the rate of spontaneous mutation was enhanced with an increase in altitude, reaching a maximum at the limit of vegetation. Naturally occurring radiation may be responsible for spontaneous mutations. Mericles and Mericles (1965) demonstrated that, in *Tradescantia*, a 2-week exposure of the plants to 0.25 mR/h increased the somatic mutation rate to 4–5 times over the spontaneous rate.

In addition to metabolites of unknown composition which have been proven mutagenic, D'Amato and Hoffmann-Ostenhoff (1956) reported an extensive list of mutagenic compounds such as sulphur-containing compounds, amines, amides, nitrogen-free acids, alkaloids, aldehydes, phenols, quinones, coumarins, etc., which occur in plants.

In vegetatively propagated plants, natural 'sports' are isolated and easily maintained through asexual propagation (grafting, layering, tubers, bulbils, etc.). Nearly 25% of the apple varieties cultivated in Canada and the USA were isolated from spontaneous bud sports. Also, in other crop plants such as potato, peach, pear, cherry, plum, banana, lemon, orange, mandarin, olive, strawberry, etc., spontaneous mutations occur and contribute to the development of the cultivated varieties. For instance, among the 1440 marketed varieties of roses, 35% originated from spontaneous mutations, and 45% of USA seed acreage of potato was recently sown with cultivars derived from spontaneous bud sports (Ancora and Sonnino, 1987). However, the rate of naturally occurring somatic mutation is too low to be used in the breeding process for the development of new varieties.

Induced Mutations

Induced mutations are similar in nature to spontaneous ones, but their frequency is much higher. Changes in chromosome structure can occur in somatic and reproductive cells, in non-dividing and dividing nuclei both at mitosis and meiosis. The primary event is chromosome breakage resulting in broken ends. The broken ends can fuse to reconstitute the original chromosome structure (restitution) or can either lose ability to fuse with other broken ends (healing), or undergo reunion.

Several types of induced chromosome aberrations can be expected: (i) a deficiency due to one terminal chromosomal break which can be defined as interstitial if two breaks occur in the same chromosome (deletions), (ii) a duplication (two breaks) of a chromosome segment (tandem or reverse) which can result from unequal crossing-over at meiosis or by two non-isolocus breaks in sister chromatids of mitotic chromosomes, (iii) a translocation (simple or reciprocal) when breaks in two different chromosomes undergo reunion, (iv) an inversion due to break events where the inverted segment of the chromosome includes the centromere (pericentric) or not (paracentric), and (v) a dicentric resulting from the reunion of two chromosome fragments, each with a centromere.

Taking into account the structure of DNA, genetic information can only result from the sequence of nucleotides. Therefore, it is possible to classify the various types of changes which can lead to an alteration of this sequence: e.g. (a) replacement of one base by another ('substitution'), (b) loss of one or several nucleotides ('deletion'), (c) addition of one or several nucleotides ('addition'), and (d) inversion of one or several base pairs ('inversion').

The reaction between DNA components and chemical mutagens is supposedly a prerequisite for the process of mutations. The particular reaction may cause two main changes:

1. substitution of one base-pair with another (mis-sense or non-sense mutations);
2. addition or deletion of one or a few base-pairs in the segment of genetic material.

Assuming that a mutation represents a change in the nucleic acids carrying the genetic information, the interactions between nucleotides and chemical mutagens are of primary importance in creating mutations. However, only the induced changes compatible with the replication of nucleic acids and having a phenotypic expression are practically retained as expressed mutations. Many mutations are 'silent' and these usually occur in genes, or part of genes that are totally inactive. They can only be detected with molecular markers or by DNA sequencing.

Gross chromosomal aberrations affect many loci and are induced with a high frequency, but rarely result in desired mutations in highly selected plants. Chromosomal changes and quantitatively inherited variation are estimated with a mutation rate of 1×10^{-2}.

Single locus changes accompanied by few or no other genetic changes are the most desirable, although difficult to be accurately classifiable. The determination of a mutation as a true intra-locus event relies upon precise genetic tests, requiring the availability of closely linked flanking markers together with studies on the fertility of the homozygous mutant and on the genetic transmission of the mutated gene. Such conditions are available for only a few, well-studied model species. Very few reliable data are available for higher plants.

Brock (1971) estimated a reasonable mutation rate for intra-locus recessive mutations in higher plants of less than 1×10^{-4} per locus and per cell, by using a

medium dose of X- or gamma-rays. For example, 'high-lysine' mutations in barley are known to occur at a number of loci; if a number of 10 loci is assumed, an expected mutation frequency of 1×10^{-3} would result, which is close to the frequency observed by Doll (1975). Similarly, if 500 loci are assumed to influence chlorophyll synthesis, and mutation of any locus will induce a chlorophyll-deficient mutant, the expected mutation frequency would be of 5×10^{-2}, which is comparable with the observed mutation frequency. In other studies, Gustafsson *et al.* (1967) determined that at least 26 loci are involved in determining the short, stiff-straw, *erectoid* mutants of barley, which explains the relatively high frequency of induced mutations for this trait.

Single dominant gene mutations occur at very low frequency. For instance, out of 1024 chlorophyll mutants induced in *durum* wheat, only five were found to behave as dominant or semi-dominant (D'Amato *et al.*, 1962). A detailed analysis of 126 independently obtained mutations revealed that only some of them were semi-dominant and very few were fully dominant (Scarascia- Mugnozza *et al.*, 1993). A monogenic dominant mutation for short straw has been obtained in *durum* wheat (Scarascia-Mugnozza and Bozzini, 1966). Dominant mutation for dwarfness has also been reported for bread wheat (Barabas and Ketesz, 1985). To our knowledge, none or very few experiments have been undertaken to clarify the nature of these rare, dominant mutations at the molecular level.

The rate of mutation that can be expected from recessive to dominant alleles is difficult to estimate. Reverse mutations are greatly influenced by the nature of the forward mutations. In higher organisms, the vast majority of the 'revertants' is rather the result of mutations at some other (suppressor) locus. However, Brock (1971) estimated 1×10^{-5} as the induction rate for dominant mutations, but this should be regarded as over-estimated, the value being more likely between 10^{-5} and 10^{-6}.

Recessive mutations will be expressed only in the homozygous condition after self-fertilization or sib-mating of the mutated plants. This situation can occur in the M_2 generation at the earliest, following self-fertilization of the M_1 plants. In self-incompatible species, sib-mating of the M_2 individuals will allow expression in the M_3 generation. Recessive mutations induced in heterozygous plants may be expressed in the form of homozygous chimaeric tissue in the M_1 generation. Dominant mutations will be expressed when heterozygous and hence can be detected in the M_1 generation.

Induction and Selection of Mutations

Physical and Chemical Mutagens

Physical and chemical mutagens are tools for enhancing and generating genetic variation by inducing changes (mutations) at the gene, chromosome and genome

levels, in nuclear and cytoplasmic organelle DNA. The inheritance of the induced changes by using X-rays was first demonstrated in fruits and ornamentals (Pirovano, 1922) and barley (Stadler, 1930). The search for more efficient mutagens and for the possibility of directing the mutation process has been -intensely continued in the following three decades (1940s, 1950s and 1960s). These comparative studies of physical and chemicals mutagens have clearly demonstrated their mode of action on the biological material, their relative effectiveness and the relation of dose/concentration for increasing the mutation rate. Chemical mutagens produce less chromosomal aberrations and rearrangements than physical ones.

An ultra violet (UV) monochromatic light source (range 250–290 nm) has limited penetration ability and can be used only for pollen or single cell layer treatments. The UV radiation induces production of pyrimidine dimers which, through excision repair of DNA during replication, leads to the formation of single-strand gaps in the DNA.

The electromagnetic radiation, X-rays and gamma-rays, penetrate deep into the tissue (depending on their energy) where they lose energy by interacting with atoms and molecules. The ionization is sparsely dissipated along the track of the particle.

Particle radiation (fast and thermal neutrons) dissipates a large number of ions along the track (densely ionizing radiation) while passing through the biological material, resulting in a high frequency of induced chromosomal aberrations. Numerous alterations of DNA are induced by radiation, including double breaks in one strand, single strand breaks, rupture of hydrogen bonds between base-pairs, breakage of phosphate-diester bonds in the sugar-phosphate backbone, and cross-linkage between and within polynucleotide strands.

Chemical mutagens are cheap and readily available; however, they are hazardous to use and to dispose of. Mutagenic effects of chemicals (iodide and copper sulphate) were first reported in the 1930s, and such compounds were later found to be weakly mutagenic on *Drosophila.* During World War II, the strong mutagenic activity of mustard gas and urethane was clearly demonstrated. Since that time a large number of chemical compounds have been described for their mutagenic activity (FAO/IAEA, 1977):

1. *Alkylating agents* [ethyl methanesulphonate (EMS), ethyl ethanesulphonate (EES), diethyl sulphate (DS), and mustard gas, dichloroethylsulphide ($S(CH_2CH_2CI)_2$)]
2. *Nitrous acid* [ethyl nitroso urea (ENU), methyl nitroso urea (MNU), etc.]
3. *Hydroxylamine*
4. *Imines and mustards* [Ethyleneimines (EI)]
5. *Azides* [sodium and potassium azide].

Transpositive and Insertional Mutagenesis

In contrast to mutations induced by physical or chemical mutagens, the insertional mutagenesis approach readily identifies mutated genes and allows the cloning of

the wild-type gene. Mutations are caused by mobile genetic elements which are integrated in a genome within or near a coding sequence of a gene. When the mobile genetic element is well characterized, it can be used as a molecular tag for the isolation of the mutated gene. Two types of molecular tags have commonly been used, namely transposons and T-DNA of *Agrobacterium tumefaciens.*

Transposon tagging

McClintock (reviewed in 1987) was the first to describe transposons in maize. The insertion of transposable elements results in gene inactivation and mutation. The function of mutated genes can be identified by phenotypic screening. The sequence of the known transposable element can be used as a hybridization probe to clone the mutated gene. The isolated mutated gene can be used to isolate the wild type allele from a genomic library of a normal plant. Transposons have been used by molecular biologists to isolate and clone genes of interest (Marks and Feldman, 1989; Gierl and Saedler, 1989).

Plants are particularly prone to mutagenesis caused by transposons, and homozygous plants for the induced mutation are quickly recovered. Transposon tagging mutagenesis in *Antirrhynum* led to the isolation of a homeotic gene *Flo* (floricaula) which regulates the morphogenesis of the flower (Coen *et al.*, 1990). Experiments were carried out in 13 000 plants containing *Tam3* transposon, grown at 15°C to allow transposons 'jumping' to new sites. Phenotypes with flower morphology changes were observed in progenies from self-pollination, and among them a *flo-613* mutant was unable to produce flowers. Cloning the mutated gene was possible by using *Tam3* DNA as a probe in Southern blotting analysis of both wild and mutated plants.

Agrobacterium T-DNA tagging

T-DNA is inserted through *Agrobacterium* infection in the plant cell genome causing mutations ('insertional mutagenesis') (Feldmann *et al.*, 1989). To isolate the gene of interest, a population of transgenic plants is screened for the desired phenotype. The mutated gene can be identified from a genomic library of mutated plants using T-DNA as a probe. In *Arabidopsis thaliana*, a homeotic gene *Ag* regulating the flower morphogenesis was obtained by T-DNA tagging (Yanofsky *et al.*, 1990). A mutant *ag-2* showed multiple rays of petals and sepals, instead of a single ray as in the wild type. The gene *Ag* (wild type) has been cloned and introduced in the mutant plant *ag-2*. Among the regenerated plants, some with normal flower morphology were found. A similar type of mutant, *ag-1*, was also obtained by EMS treatment in *Arabidopsis* (Feldmann, 1992).

Somaclonal Variation

Genetic variation which occurs in tissue cultures and in plants regenerated through indirect morphogenesis, is called 'somaclonal variation', which may be

either genetic or epigenetic in nature (Ahloowalia, 1986). The origin of somaclones can be traced by the genetic changes already present in the cells of the cultured tissue or can be induced by the mutagenic action of the culture media (true heritable changes), or by stress conditions during *in-vitro* culture (possibly epigenetic variation, Brown, 1991). The contribution of somaclonal variation to crop improvement is discussed in Section 1 of this book.

Mutation breeding

Any proposal to use induced mutations in plant breeding must consider the likelihood of success and the efforts required to obtain the desired genotype when compared with other techniques. The likelihood of success cannot be predicted, although it can be estimated on the basis of the genetic control of the character to be improved and the breeding system of the species.

A number of basic principles must be taken into account while planning a strategy for a successful mutation breeding programme. Unfortunately, too often the breeder adopts a strategy aiming at a desired phenotype, but with little or no knowledge of its genetic base. When the desired traits are controlled by recessive genes, the induction of mutations is worthy of consideration. If the gene(s) controlling the desired character is available in a genotype closely related to the cultivar, gene transfer by conventional crossing would normally be favoured. However, there are examples of rapid acquisition of a single desirable character by means of mutations.

Conventional breeding strategies may in fact be impractical in all cases where the gene for a desired trait is closely linked to genes with undesirable effects, or if undesirable pleiotropic effects are known to affect the trait, or whenever interspecific or inter-generic crosses are involved. In all these cases, induced mutations may represent a convenient approach. On the other hand, if there is no known genetic source for a desirable trait, induction of mutations is the only possible approach. In general, the choice between induction of mutations and gene transfer for simply inherited traits, is largely determined by the ease with which the desired gene can be incorporated from another genotype by conventional crossing, and by the ease of inducing the desired mutation. In this regard, the induction of a recessive mutation is a much more likely event than the induction of a dominant one (Brock and Micke, 1979).

In self-fertilizing species, the response of quantitatively inherited characters to random mutations will depend on: (a) the number of genes involved, (b) the relative portion of genes with positive and negative effects, (c) the extent to which the genes of the parental genome operate as a balanced set, and (d) the correlation with linkage or pleiotropy. In general, random mutations will increase the variation of all quantitatively inherited characters and shift their means away from the direction of previous selection. Therefore, the efficiency of selection for the desired mutant is generally lower than that for a specific trait which is controlled by a single gene.

When the species to be improved possess a narrow genetic base due to intensive plant breeding, substantial variation is likely to be generated by either muta-

tion or intervarietal hybridization. Consequently, the choice of a suitable method for inducing variation will be largely determined by the amount of variation already available for a trait and by the nature of the trait to be improved. When mutant identification is relatively easy, as in the case of early flowering, induced mutations become an attractive means to generate genetic variation. Similarly, when mutant selection is difficult and expensive, as in the case of yield, hybridization between high-yielding varieties is a better option because high-yielding segregants are usually recovered with a higher frequency, and their genetic background is not disturbed by random mutations which could negatively affect other important traits (Brock and Micke, 1979).

In the case of well-adapted varieties, limited or no variation is expected to be generated by inter-varietal hybridization with similar high-yielding and well-adapted genotypes. In these situations, substantial variation can only be generated by wide hybridization or by mutation; in this regard, mutation breeding is less likely to disturb the genetic background of a generally well-performing variety.

In general, induced mutations offer less prospects for the improvement of cross-pollinated than for self-pollinated species. This is partly because of the difficulty of selecting, incorporating and maintaining recessive mutations in cross-pollinated plants, and partly because, in cross-fertilizing species, the main problem is the handling of existing variation rather than its lack *per se*. However, the nature of genetic variation is more important than its amount; therefore, improvement programmes of cross-fertilizing species have successfully used the variation induced by mutagenesis.

Many vegetatively propagated species, which include several horticultural and ornamental plants of economic relevance, are highly heterozygous and often polyploid or aneuploid. The variation obtained by crossing is so wide that there is little chance to select for improved progenies with desirable traits and, at the same time, retain the general characteristics of the parental variety. In these plants, mutation breeding offers the advantage of increasing the frequency of mutations (over the spontaneous rate) by inducing changes (mostly recessive) in one or a few traits without altering the outstanding characteristics of the original variety (Donini *et al.*, 1990). In the vegetatively propagated species which are either apomictic or sterile, hybridization is not an option, and induction of mutation is the only method for improvement.

Technical Aspects of Mutation Breeding

The technical aspects of mutation breeding deal with the choice of the mutagen and dose, the starting material and population size, the handling of mutagenized material, and the selection of suitable breeding methods (FAO/IAEA, 1977).

The type of mutagenic treatment and dose must be possibly matched with the genetics of the desired trait and the breeding system of the species. In general, both physical and chemical mutagens should be used at dose/concentration of LD_{50} allowing the treated material to produce M_2 progenies. This allows one to

generate a high-rate, wide spectrum of induced mutations, while retaining a population of a reasonable size from which to recover useful mutants.

It should also be remembered that densely ionizing radiations (thermal and fast neutrons) usually cause more chromosomal aberrations, while chemical mutagens produce high rates of gene mutations. Furthermore, a suitable dose/concentration has to be determined before hand because differences in plant sensitivity to mutagenic treatments exist, and depend on: (a) environmental conditions of the treated material (e.g. water content, oxygen, temperature, etc.) and (b) biological factors (degree of ploidy, genetic differences, etc.).

In sexually propagated plants, seeds are the favoured material for mutagenic treatments because of their ease in handling. However, seed is a multicellular organized structure containing a well-differentiated embryo with an apical meristem, a number of leaf primordia and the initial cells (two or more) of the sporogenous tissue. A mutation induced in a single cell of the sporogenous tissue will give rise to a chimaeric M_1 plant. The size of the chimaera is the result of competition between mutated and non-mutated cells (diplontic selection) which occurs during cell divisions, and of the extent of their participation in the formation of the reproductive organs. An induced mutation will normally be present in the M_1 plant in a heterozygous state. It will be expressed only in the M_2 generation, provided that it resides in cell(s) of the sporogenous tissue, from which the gametes are formed, and that no unfavourable competition against mutated gametes (haplontic selection) takes place during fertilization.

Mutagenic treatment of pollen grains, female gametes, or both, offers a great advantage over seed irradiation in that completely heterozygous M_1 plants without chimaerism are obtained for any induced mutation. The disadvantage of male gametes irradiation resides in the rather short viability of the pollen. Other materials can be used for mutagenic treatments including: seeds of a F_1 cross between two varieties aiming at enhancing the frequency of translocations between the genome of the two parents; microspores which allow the recovery of haploid mutated cells from which dihaploid homozygous mutated plants can be recovered following chromosome duplication, and *in-vitro* suspension cultures of single somatic cells capable of plant regeneration (Maluszynski *et al.*, 1995).

The breeder has to choose the variety to be mutagenized with the knowledge of its genetic characteristics, of the uniformity of the material and the possible existence of the desired trait in the pool of available germplasm. In practice, the chosen variety should be well adapted, have outstanding agronomic performance and require the least number of genetic modifications (e.g. short and stiff straw, lodging resistance or resistance to a specific disease).

The amount of material to be treated (seed, pollen, microspores, etc.) and consequently the number of M_1 and M_2 plants to be grown, are important aspects of applied mutagenesis. In fact, a minimum number of materials ensuring a sufficient likelihood of success would be preferable for economic reasons The required minimum number of M_1 plants and M_2 progenies can be calculated considering the expected mutation frequencies and types of mutational changes, in addition to the breeding system of the species (Brock and Micke, 1979). However, mutation

frequencies vary greatly depending on the locus (or number of loci), type of the mutational event and mutagenic treatment. Assuming a mutation rate and setting a level of probability for the occurrence of at least one mutation, the number of treated cells that have to be examined can be calculated (Brock and Micke, 1979). Several studies indicate that different loci do not respond in the same way to a mutagenic treatment, hence to predict *a priori* the mutability for a given trait is rather difficult. The degree of heterozygosity and the ploidy level of a plant are also important in mutagen response.

In the case of single cell organisms, no problems are found in the separation of single cells after mutagenesis, and isolated single colonies can be subjected to selection. Mutagenic treatment of single cells, such as pollen grains, eggs and protoplasts, results in the selection of genetic or somatic mutations in M_1 or M_2 generations, depending on the original genetic situation (homozygous vs heterozygous in the case of protoplasts). However, in multicellular organisms such as higher plants, the organ that is exposed to mutagenesis, for instance seeds, shoot tips and other tissues, is usually a multicellular one; therefore, the progeny from a mutated cell will represent only a portion of the first generation growth, and the M_1 plant will be chimaerical. For this reason, it is important to know the number of cells in the multicellular organ (seed, meristem, primordia, etc.) which are exposed to the mutagenic treatment and which will contribute to the next generation ('genetically effective cell number') (Brock and Micke, 1979). The 'genetically effective cell number' has been estimated for seeds of a number of species, and is usually between two and ten (D'Amato, 1964b; Li and Redey, 1969; Beard, 1970).

In practice, the population size for mutation breeding experiments is based on the frequency of a mutational event for a specific locus of interest. Assuming a 90% probability of success in recovering a mutant occurring at a frequency of 1×10^{-3} test units (e.g. spikes, fruits, branches), and that each plant grown is expected to produce three units, the amount of material to be treated, if the M_1 has a 80% survival rate, is about 600 seeds. As an indication, lodging-resistant mutants have been frequently obtained in chemically or physically treated plants such as oat, barley, bread wheat, *durum* wheat and rice, by treatment of at least 1 200 seeds, whereas resistance to viruses and diseases are much less common, requiring a 10–20 times increase in the size of treated sample. In addition, the degree of chimaerism in the M_1 plant, the genetic characteristic of the trait (simply or quantitatively inherited), the mutagen dose/concentration, the segregation ratio and the selection accuracy should also be taken into account in mutant recovery.

Handling the Treated Material

Mutagenic treatments induce some sort of physiological and genetic injury to the M_1 plants; therefore, these should be grown in a healthy environment and in isolation in order to prevent outcrossing. The M_1 plants can be isolated either by

appropriate distance or by bagging, and they can be harvested by the following procedures:

1. Single spike, branch or plant progeny. In monocots, e.g. cereals and grasses, the maximum potential for induced genetic variation is in the primary tillers, which arise from the already-differentiated bud primordia present in the seed embryo. In dicots, largely self-pollinated plants such as beans, peas, sunflowers, tomato, etc., the main branch of the M_1 plant is harvested.
2. Single or multiple seed bulk method. This is useful when the probability for the occurrence of a single mutant offspring within the progeny of a fruit developed from a mutated tissue is higher than the frequency of the particular mutation in treated samples from the whole population.
3. Mass bulk method. This is applicable when land and resources are cheaper than labour.

Similarly, M_2 populations could be managed according to one of the following procedures: (i) population bulk system; (ii) ear to row bulk; (iii) single seed or multiple-seed bulk; (iv) plant to row; and (v) ear, branch, pod, fruit to row.

Dominant mutations can seldom be detected in the M_1 generation under special circumstances. In such cases, the number of cell progenies (230 000 to 2 300 000), represents the minimum number of M_1 plants that have to be screened (Brock and Micke, 1979). Lundqvist and Lundqvist (1991) reported dominant resistance to barley mildew race D1, isolated after mutagenic treatments in four barley varieties: Bonus, Mari, Pallas and Kristina.

Recessive mutations can normally be detected in the M_2 and M_3 generations after selfing the M_1 plants. For seed treatment, if we assume five initial cells per treated seed, a total of 10 000 seeds will be required. The number of M_2 families required for the expression of the recessive mutations is determined by the segregation ratio, which should normally be 1/4 but decreases to 1/20 when the initial number of cells is five.

Methods of Selection

Selection of induced mutations is an important step in mutation breeding, and is far more difficult than the induction itself. The procedures of selection could be divided into two categories: (i) mutations detectable on a single plant basis by visual inspection combined with appropriate screening procedures (e.g. artificial infection, induction of lodging, temperature stresses, etc.); and (ii) mutation detectable on a plant-progeny basis. Mutation invisible to the naked eye (e.g. quantitative traits: yield, seed protein, etc.) can be identified only through biochemical or biometric investigations on the whole population basis.

The visual selection method for identifying mutant phenotypes is the most effective and efficient. The easily detected characters are: earliness, plant height, colour changes, non-shattering, disease resistance, etc., while mechanical or physical methods of selection are particularly efficient for characters such as seed size, weight, density, etc.

Chemical, biochemical, physiological, physico–chemical and various other screening procedures may be needed for the selection of mutants, e.g. (i) insensitivity to gibberellin can be identified by spraying gibberellin solution onto seedlings or by soaking seeds in a gibberellin solution, then selecting those seedlings with little or no response; (ii) low alkaloid content mutants may be identified using colorimetric tests on M_2 or M_3 seeds; (iii) resistance or tolerance to herbicides is screened following chemical treatment of M_2 seedlings; (iv) resistance or tolerance to diseases is tested by treating the M_2 seedlings with infectious materials such as fungi, spores, cultural filtrates.

Progeny tests are essential for the identification of mutant lines which will be useful and therefore chosen for re-selection in the M_3 generation. Further progeny tests may be necessary to stabilize a potentially useful mutant. In a number of cases, M_3 progeny tests may be essential for the detection of mutants, particularly of those not so readily discernible among single plants.

The value of *in-vitro* selection of mutagenized calli, cell suspensions or protoplast cultures, resides in its ability to select variants from large numbers of totipotent cells in small culture flasks. Its efficiency derives from its ability to select cells that are able to regenerate plants rather than to select at the whole plant level (Baenziger, 1985). Obviously, selections are restricted to the type of mutations that can be detected at the cellular level such as tolerances to salinity, mineral stresses, herbicides, disease-related toxins, etc.

Great interest resides in selection at the haploid level due to the gametic number of chromosomes that allows expression of both dominant and recessive gene mutations.

When a mutant appears to be stable and uniform, it starts the phase of seed multiplication and field testing. In the agronomic evaluation phase, the mutant(s), the mother variety and other outstanding varieties are grown under similar conditions for comparison. Valuable mutants will finally be released as new varieties to farmers. Figure 1 shows the mutation breeding scheme for the improvement of seed-propagated plants.

The recovery of mutations in vegetatively propagated plants is different from that of seed-propagated plants, in which a mutation is recovered following gametic recombination and segregation. In vegetatively propagated plants, a mutation event induced in a somatic cell (somatic mutation) of any cell layer of the shoot meristem or in the axillary primordia will be transmitted and eventually expressed in somatic descendants (clones). Moreover, in shoots developing from the treated organs such as buds, the mutated cell will develop into a sectorial or mericlinal chimaera through cell divisions and competition with unmutated cells. A mericlinal or sectorial chimaera is unstable, and only through growth and clonal propagation could it form a periclinal chimaera, or a completely uniform mutated organ (homohistont). Therefore, the detection and isolation of induced somatic mutations may present considerable difficulties, and an appropriate method has to be used both for *in-vivo* and *in-vitro* clonal propagation.

The basic principle of this method is based on forcing the growth of the axillary buds along the M_1V_1 shoots, particularly those of the basal and median zone, by

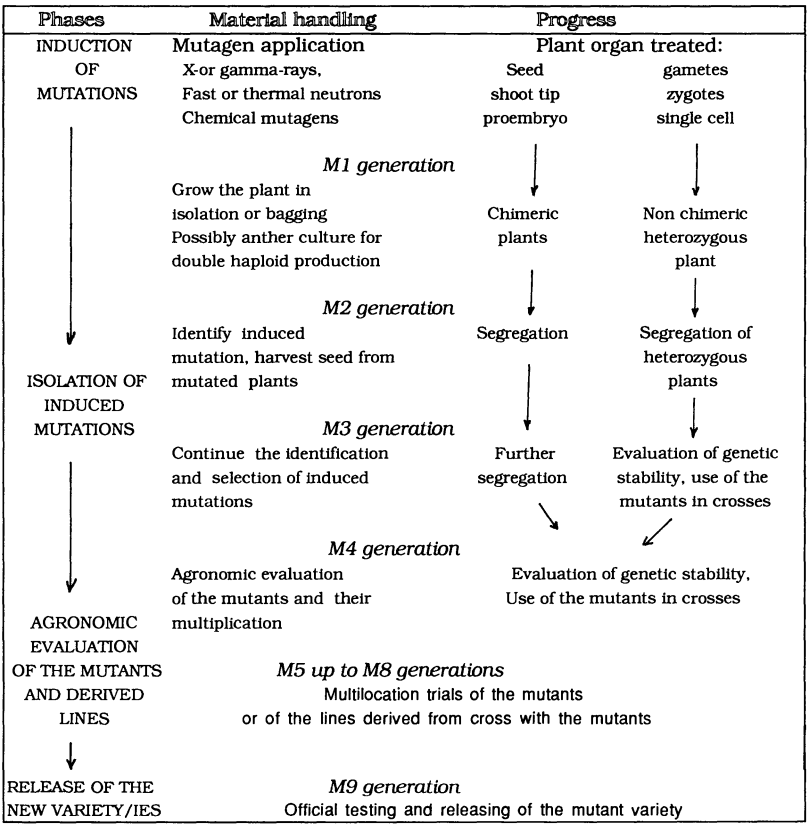

Figure 1. Mutation breeding scheme for the improvement of seed-propagated plants (modified from: Micke and Donini, 1993)

pruning or decapitation of the M_1V_1 shoots ('cutting back system') or by grafting the axillary buds into new rootstocks. A similar procedure will be used in handling the M_1V_2 generation. Figure 2 shows the mutation breeding scheme for the improvement of vegetatively propagated plants.

Mutation breeding combined with *in-vitro* cultured materials such as shoot tips, nodal cuttings and adventitious shoots, and the micropropagation of axillary buds, is considered as a valid approach for the isolation of somatic mutations. In contrast to the *in-vivo* method, *in-vitro* culture results in a quick dissolution of the chimaera and in the recovery of genetically stable mutants (Sonnino *et al.*, 1986; Ancora and Sonnino, 1987). Figure 3 shows the mutation breeding scheme for the induction and selection of somatic mutations through *in-vitro* culture.

The isolated mutants in the M_1V_2 and/or in M_1V_3 generations can be evaluated for their stability, and multiplied to test their agronomic performance. *In-vitro* mutagenized meristems can be screened directly *in vitro* for a number of favourable attributes, including resistance to abiotic and biotic stresses (Sonnino,

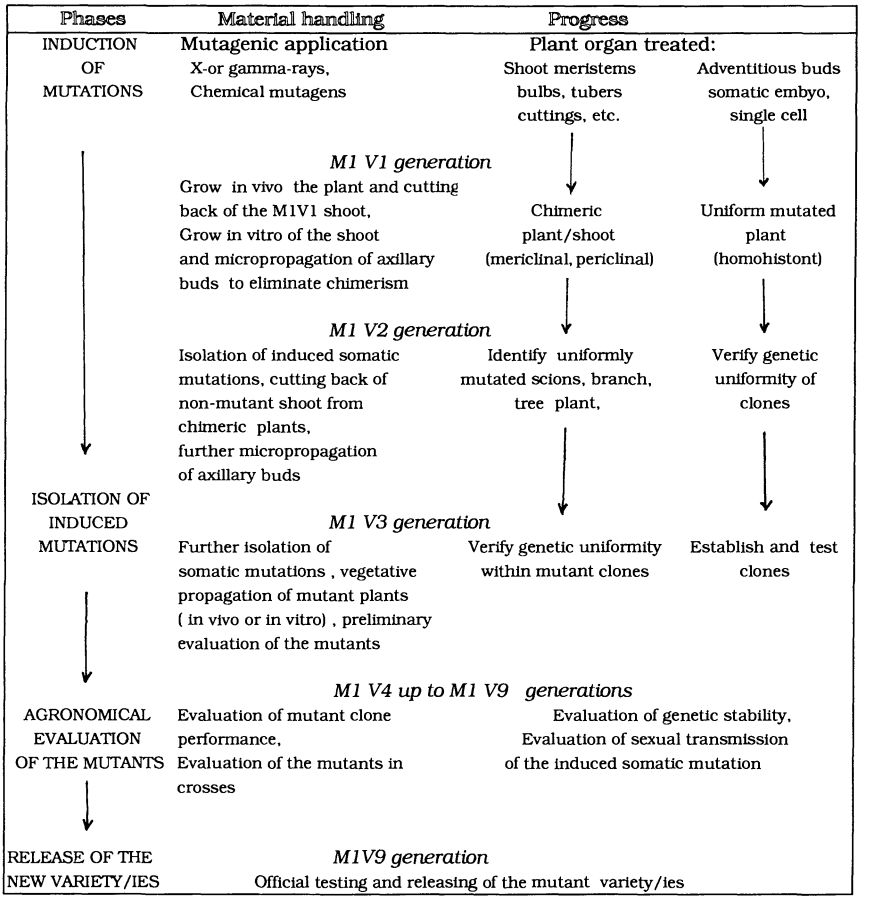

Figure 2. Mutation breeding scheme for the improvement of vegetatively propagated plants (modified from: Micke and Donini, 1993)

1991). *In-vitro* selection allows the handling of large populations under controlled and reproducible conditions. Improved mutants are then released to growers.

Uses of Induced Mutations

In Plant Breeding

The direct use of mutations is a valuable approach especially when the improvement of one or two easily identifiable characters is desired in an otherwise well-adapted variety. The main advantage is that the basic genotype of the variety is usually altered slightly as opposed to procedures involving hybridization of two distinct varieties. A mutation for a desired character(s) can be incorporated in a

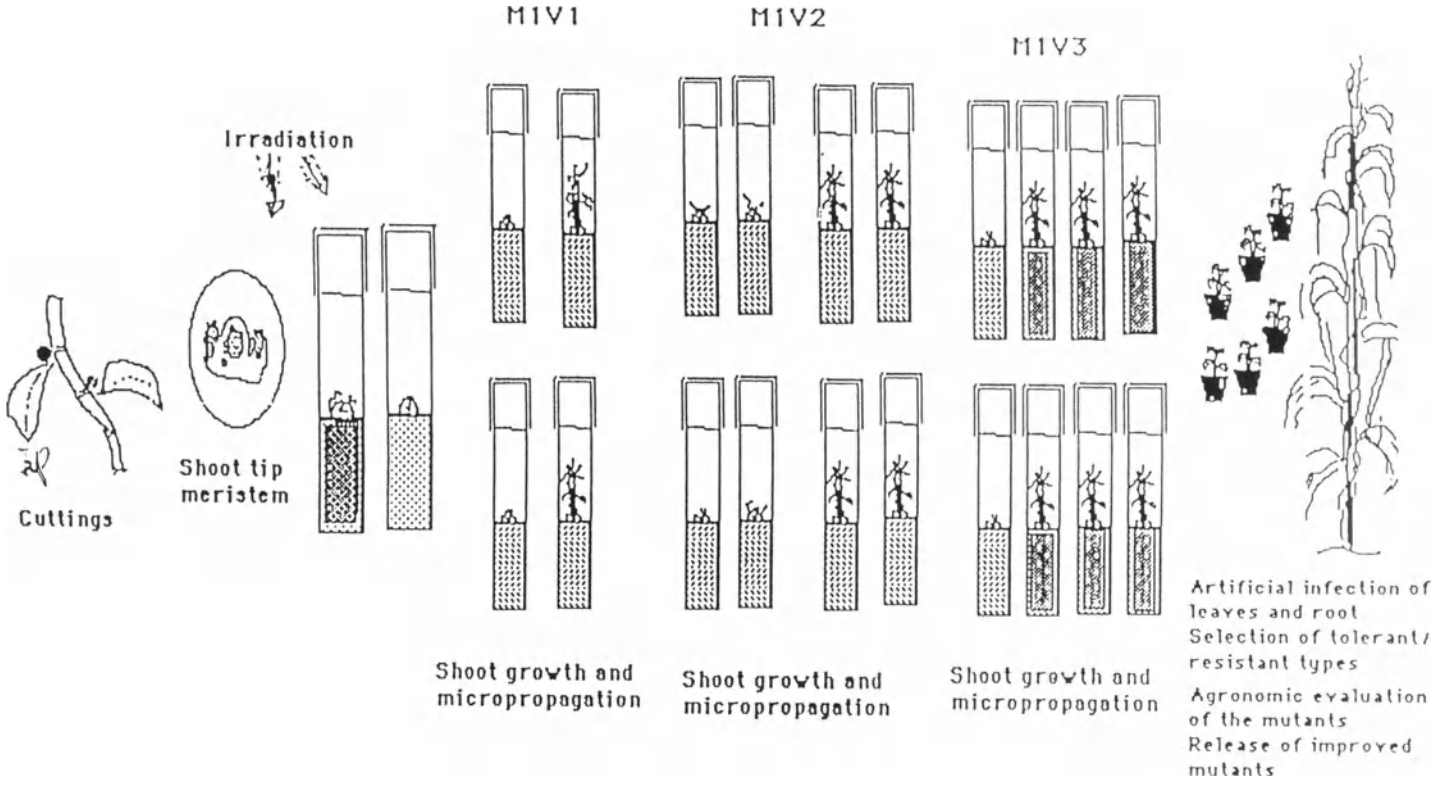

Figure 3. Mutation breeding scheme for the induction and selection of somatic mutations through *in vitro* culture (from B. Donini, ENEA, Division Biotechnologies and Agriculture, Rome)

variety, thus reducing the time required to breed an improved variety with the conventional hybridization method. Several cultivars derived from direct utilization of induced mutants have shown that traits such as short straw, earliness and resistance to certain diseases, can be introduced in otherwise well-adapted varieties without significantly altering their original attributes.

The indirect use of mutants may involve: (i) crossing different mutants from the same parent variety, (ii) crossing different mutants from different parent lines, and (iii) crossing two varieties apparently carrying the same mutation in the same parent variety.

When undesirable characters are observed in a mutant, it is advisable to backcross the mutant to the parental line and to select for individuals with the desired mutation and free from other undesirable, induced mutations. Because of the nearly identical genotypes of the parents, one or two backcrosses should normally be sufficient to clean the mutant from undesirable characters caused by additional mutations.

In the long run, the use of induced mutants in hybridization breeding programmes is probably more important than the direct use of the mutants; in fact, the relative number of varieties derived from cross-breeding involving induced mutants has steadily been increasing with time. When two mutants from the same variety (differing in two mutant genes) are crossed, a small F_2 population is needed to obtain a recombinant, because of their generally similar genotypes (Gaul, 1963). However, some evidence exists that intercrossing of mutants can give new transgressive variation (Scarascia-Mugnozza *et al.*, 1972).

In vegetatively propagated plants, mutants are usually used directly as improved varieties. The crossing of the mutants with the original variety or with other varieties in order to transfer the mutation will be successful only if the induced mutation is located in the sporogenous tissue.

Mutations for Plant Domestication

Mutations have played an important role in the domestication of wild species (for an extensive review see FAO/IAEA, 1989). Under natural conditions, evolution results in the accumulation of genes which are advantageous to the survival and reproduction of the wild plants. Many of these wild genes are not desirable in agricultural situations.

The activity of wild plant genes, which are usually in the form of dominant alleles, can be deleted or altered by mutation to their recessive alleles, or can be suppressed by mutation of other modifier or regulator genes. Removal or suppression of undesirable features such as shattering seeds, fragile spike, dehiscent fruits, presence of toxins, spines or thorns, is the first step in the adaptation of plants for human use.

Induced mutations will play an important role for developing cultivars from existing crops, which present novel and valuable traits for new uses ('second cycle domestication'), or to domesticate new plant species which have so far been neglected by humans ('primary domestication') (Ashri, 1989). Examples for

new uses of already domesticated species are soybean (*Glycine max*), previously grown as a vegetable and now as a protein and oil crop, and lupins (*Lupinus* spp.) from green manure to seed crop. Fairly recent examples of primary domestications are rubber (*Hevea brasiliensis*), African oil palm (*Elaeis guineensis*) and kiwi (*Actinidia chinensis*).

Perspectives are foreseen for plants yielding oils, carbohydrates and other valuable products for industrial uses, and for new fruits, vegetables, flowers and ornamentals offering better quality and attractiveness, as well as new protein grain legumes for human consumption and animal feed. As an example, many potentially valuable high-protein grain legume species exist that have undesirable morphological traits, antinutritional factors and toxins which hamper their regular use, and they could well be domesticated by mutation breeding. However, the utilization of induced mutations in plant domestication has so far been reported in a limited number of cases. In spurge (*Euphorbia lagascae*), a wild Mediterranean plant rich in vernolic acid (58–62%), which is used in oleochemistry, several mutants for indehiscent capsule and high number of seeds per capsule were obtained following EMS seed treatment (Villalobos *et al.*, 1994). Chemical mutagenesis has been efficiently used for the domestication of *Cuphea*, a potentially interesting plant for the production of medium-chain fatty acids, and mutants for no sticky hairiness, monoculm stems and determinate growth have been obtained (Röbbelen and von Witzke, 1989). Unfortunately, fruit indehiscence, that was the most desired trait, has not been induced.

Understanding Biochemical/Genetic Functions of Genes

Induced mutations are being used to gain knowledge in biochemical genetic analyses of the biosynthetic mechanisms responsible for many important traits, including nitrogen and carbon assimilation and conversion (Ichii *et al.*, 1993; Koornneef and Kendrick, 1994).

Mutants may uniquely permit analysis focused on very small, perhaps truly single gene differences. Abscisic acid-deficient mutants of sunflower were used to elucidate the correlation between capacity to synthesize abscisic acid and drought response (Fambrini *et al.*, 1995). Induced mutants have already played a vital role in advancing our knowledge on photosynthesis (Koornneef and Kendrick, 1994). Several hundreds of induced chlorophyll-deficient mutants of barley made possible a unique series of analyses of the photosynthetic apparatus of higher plants as well as of chloroplast structure biosynthesis (Lundqvist and Lundqvist, 1994).

There are several applications of mutants in basic research and particularly in the identification of changes in a single base-pair in DNA, especially in molecular plant physiology and genetics. Many molecular studies currently rely on the induction and identification of mutants in model species such as *Arabidopsis*, for the construction and subsequent saturation of genetic maps, and for the understanding of developmental genetics and biochemical pathways (Sun *et al.*, 1992). Once identified and isolated, the genes that encode agronomically important fea-

tures can either be introduced directly into crop plants or used as probes to search for similar genes in other crop species.

The role of genes involved in the process of controlling the regular formation of bivalents at meiosis in allopolyploids such as wheat, has been extensively investigated using mutations at these loci. Studies on double mutants for the two most potent suppressor genes (*Ph1* and *Ph2*) controlling homoeologous pairing in wheat, have been carried out in wheat itself and in hybrids with alien Triticeae in order to elucidate their effects on chromosome pairing (Ceoloni and Donini, 1993).

The study on the molecular nature and on the localization of mutations in a genome will be enhanced in the future by novel and powerful DNA fingerprinting techniques. In particular, molecular markers such as AFLP (amplified fragment length polymorphism) (Zabeau and Vos, 1993), seem to offer the greatest potential for targeting mutations and for uncovering the effects of methylation on gene function (Donini *et al.*, 1997).

Major Achievements

Mutation research bloomed after the UN Geneva Conference in 1966 on 'Peaceful Application of Atomic Energy', when the main emphasis was given to the radiobiological aspects of both physical and chemical mutagens, their relative effectiveness and their interaction with biological matter. In the late 1960s, greater interest was paid to mutation breeding technologies to be applied in breeding of both seed and vegetatively propagated crop plants. At the end of the 1960s, 117 varieties were developed by mutation breeding (Sigurbjornsson and Micke, 1969).

In the early 1980s, the list of mutant varieties had greatly increased to 600 (Micke and Donini, 1982) and by the end of the decade it reached 1363 entries; these include direct mutant varieties and varieties originating from crosses with induced mutants. In the last two decades, the number of varieties resulting from crosses involving mutants has sharply increased.

In the 1990s, cereals and ornamentals mutant varieties were far more numerous (586 and 406, respectively) than those of other categories of plants. Mutant varieties of the other plants included grain legumes (86), oil seeds (83), fruit crops (40), vegetables (40), fibre crops (28), forage crops (25), root and tubers crops (11), and other (17). Table 1 shows the number of varieties of seed and vegetatively propagated plants developed through mutation breeding up to 1993. A much greater number of mutant varieties was developed in Europe and Asia (42% and 44% respectively), as compared to other geographical regions such as North America (7%), Latin America (5%) and Africa (2.0%).

The major improved characters reported for the released mutant varieties of seed-propagated crop plants have been: increased yield, reduced plant height, early maturity, resistance to pathogens, improved seed quality and improved adaptability (Table 2) (Donini *et al.*, 1984), whereas the mutants of vegetatively propagated crops involved changes in flower colour, plant architecture (flowers

Table 1. Varieties of seed and vegetatively propagated plants developed through mutation breeding.

Group of plants	Direct use of mutant	Varieties from crosses with mutants	Total	Main crop plants
Seed-propagated plants				
Cereals	350	236	586	Rice, bread, wheat, barley, *durum* wheat, oat, maize
Grain legumes	62	24	86	Soybean, peanut, bean, mungbean, chickpea, cowpea
Oil seeds	71	12	83	Rape seed, sunflowers, castor bean, mustard, linseed
Vegetables	35	5	40	Lettuce, tomato, onion, sweet pepper, pea, Chinese cabbage
Fibre crops	23	5	28	Cotton, jute, fibre flax, ramié
Forage crops	23	2	25	*Trifolium, Lolium*
Others	12	5	17	Tobacco, serradella, lespedeza, buckthorn
Vegetatively propagated plants				
Tuber and bulb plants	47		47	Dahlia, tulip, sweet potato, potato, lilium, polyanthes
Potted plants	80		80	Begonia, streptocarpus, azalea, achimenes
Cut flowers	240		240	Chrysanthemum, rose, alstroemeria, carnation
Other ornamentals	27		27	Portulaca, bougainvillea, weigela, forsythia
Fruit crops	32		32	Apple, sweet cherry, sour cherry, black currant, citrus, pear
Other crop	20		20	Peppermint, sugarcane, mulberry, turmeric

(Data base Joint Division FAO/IAEA, Plant Breeding and Genetics Section, Vienna, 1990)

and ornamentals) and period of flowering or fruit ripening, fruit colour, reduced plant size, apireny, etc.

The major successes were obtained in barley (Sweden, Denmark, Czech Rep., etc.), rice (China, India, Japan, Pakistan and the USA), *durum* wheat (Italy, Austria and Bulgaria), cereals, oil crops and legumes (India and China), flowers and ornamental plants (Netherlands and France). Few examples in specific crops underline the economic importance of the released mutants. In *durum* wheat, lodging susceptibility and straw weakness, particularly under high fertilizer input, were the main reasons for substantially lower yields compared to those of bread wheat. The extensive *durum* wheat mutation breeding work carried out in Italy resulted in 11 registered varieties; six of these originated from the direct use of induced mutants, the remainder from mutant cross-breeding.

Among the released varieties, 'Creso' became the leading Italian variety with the highest percentage of certified seed. Creso has high and stable yield capacity, good adaptability and grain quality. The cultivated area under Creso increased continuously during the past 15 years, reaching more than one-third of the total Italian *durum* wheat cultivated area. Estimating a production increase of 0.9 ton/ha over that of other varieties, it is possible to calculate a total yearly gain for Italy of 400 000 tons, equivalent to US$180 million per year (Scarascia-Mugnozza *et al.*, 1990, 1993).

Mutation breeding of rice was successfully performed in California (USA) by Rutger (1990) and resulted in the development of several semi-dwarf varieties, with added value to rice farmers which was estimated in the early 1980s to be

Table 2. Improved characters reported for mutant varieties in seed propagated crop plants.

Character	Cereal crops	Other crops	Total
Increased yield	248	131	379
Plant architecture			
Reduced plant height	289	47	336
Other characters	94	84	178
Resistance			
Pathogens	186	53	239
Pests	6	8	14
Early maturity	164	88	252
Seed characteristics			
Morphology	40	29	69
Quality	115	61	176
Other characteristics			
Adaptability	82	39	121
Threshability	10	9	19
Easy harvesting		4	4
Cold tolerance or winter-hardiness	25	6	31

(from A. Micke, FAO/IAEA, Plant Breeding and Genetics Section, IAEA, Vienna)

US$20 million. The most significant semi-dwarf mutant was Calrose 76, obtained from Calrose, a well-adapted, tall, high-yielding *japonica* variety. The mutant Carlose 76 was directly released in 1976 as the first mutation-induced semi-dwarf variety, and it was used as a semi-dwarf donor in crosses with other rice varieties. The success of rice mutation breeding in California relied on the fact that semi-dwarf mutants were induced in *japonica* types which were well adapted to the local conditions. Semi-dwarf donors of the *indica* type were available at the time, but lacked adaptability and grain quality for the USA market.

Crop improvement through mutation breeding has been very successful in China. More than 325 mutant varieties of several crop plants have been released for commercial production or used in cross-breeding. These are grown on approximately 9 million hectares, making a substantial contribution to the economy of the country. The highest number of released varieties obtained by mutagenesis is in cereals (220), oil crops (33), fibre crops (6), vegetables (6), fruit trees (7), forage crops (6) and ornamentals (43). The estimated output of cereals, fibre and oil seed mutant varieties has increased from 3 to 4 million tonnes per year. The outstanding mutant varieties of rice, cotton and soybean received the National Invention Awards (Lin, 1990).

Mutation breeding in the former USSR led to the development of 366 mutant cultivars (winter wheat, barley, white lupin, maize, etc.) of which 134 were released during the period 1977–1992 (Salnikova, 1993).

In Japan an extensive mutation breeding programme was performed over the past 40 years, resulting in the development of 12 cereal mutant varieties, five food legumes, nine industrial crops, seven vegetables and 18 ornamentals. In rice, six direct mutant varieties and 33 varieties from crosses involving mutant lines have been obtained. In particular, a semi-dwarf and stiff mutant 'Remei', has been extensively used as a parent in cross-breeding, resulting in the release of six varieties (Kawai and Amano, 1991). These achievements have led the country to build a gamma-field which is still in operation.

Over the past three decades, more than 200 new cultivars of cereals, grain legumes, oilseeds, fibre crops, vegetables and ornamentals have been obtained from mutation breeding in India. The new varieties are grown over large areas in different parts of the country, providing both economical and social benefits (Bhatia, 1990).

In the vegetatively propagated plants, more than 446 mutant varieties, of which almost 90% are flowers and ornamentals and the remaining fruit trees and others (sugarcane, peppermint, mulberry, etc.), have been released during the past three decades. However, their economic value is difficult to calculate. In the Netherlands, the contribution of flowers and ornamental mutants has been substantially high; in *Chrysanthemum* alone the mutants of cv. Horim gave an estimated economic value of US$95 million in 1978 (Broertjies and van Harten, 1978).

In the USA, the induced peppermint (*Mentha piperita*) mutants resistant to *Verticillium* wilt contributed to mint oil production, worth US$20 million per year.

Recent mutation breeding research is focused on developing new varieties with improved qualitative characteristics of crop plants for food, feed and agro-industry (e.g. oil and seed protein composition, nutritive value of the products, nutritional composition). Some recent examples are presented and discussed below.

Earliness

Six mutant lines of rice heading 8–46 days earlier than the original cultivar Norin 8 were selected following X- and gamma-rays, 5-bromodeoxyuridine (5-Bdu) and 2-aminopurine treatments (Yokoo and Okuno, 1993). The genetic analysis showed that, in two mutants induced by 5-Bdu and gamma-rays, the recessive mutations occurred at the same *Lm* locus which controls photoperiod sensitivity. Another mutant carried a different mutation responsible for earliness at the locus *Se-5(t)* on the sixth chromosome.

In rapeseed (*Brassica napus*), Thurging and Depittayanan (1992) reported the induction of early-flowering mutants in the highly inbred TB8 line of spring rape, obtained from seed treatment with EMS (0.25%, 0.50%, 0.75%, 1.00%, 1.25% and 1.50% for 12 h). The two induced mutations, one recessive and one dominant, caused M_3 plants to flower at least 20 and 59 days earlier than the parental line TB8, respectively. These mutants could be particularly valuable for improving oil-seed rape yields in low-rainfall areas.

Earliness seems to be easily obtained by means of mutation breeding in a number of plant species (Donini *et al.*, 1984) and the character is easily identifiable by visual selection.

Changes in Morphophysiological Characteristics of the Plant

In castor (*Ricinus communis*), Chauhan *et al.* (1992) induced a 'femaleness-plant' change after irradiation of dry seeds of cv. Aruna with 75, 100, 125 and 150 Krad gamma-ray doses. Among the 723 M_3 plants grown, three female mutants were identified. Two mutants originated from 100 Krad and one from 125 Krad gamma-ray doses. The three mutants were perfect females and produced only female flowers. The mutants were almost similar to the control plants for height, number of lateral branches and racemes, and when they were crossed with the control, the F_2 showed 3:1 segregation for normal hermaphrodite and female plants, which suggests that a single recessive gene controls the perfect femaleness in castor.

Narasimha Chary and Bhalla (1988) induced male sterility in pigeon pea (*Cajanus cajan* (L.) Millsp.) after seed treatment of line ICP 7295 with 0.1%, 0.2% and 0.3% EMS for 4 h. The male sterile mutant was recovered in the M_2 population and was characterized by long narrow leaves, slender stem and shrunken and irregular-shaped pollen grains, while pod setting was normal. As a result from crossing the mutant to the parent line, it was shown that the mutant

was female fertile. The F_1 plants were fertile, indicating that the male sterility character was recessive. The F_2 plants segregated into 69 fertile and 21 male sterile plants.

Mutants for plant types, flowering time and seed size were induced in black gram (*Vigna mungo* L. Hepper) with Co^{60} gamma-rays and EMS seed treatments (Thakur and Sethi, 1993). Most of the mutants behaved as recessive.

In lentil (*Lens culinaris* Med.), dry seeds of three cultivars LL-78, Pant L.639 and Sehore 74-7 were irradiated with 5, 10, 15 and 20 Krad gamma-rays (Sinha and Chowdhury, 1991). From 20 Krad treated line LL-78, one semi-dwarf mutant from the M_2 population was late in flowering and maturity. In M_3, this mutant segregated for plant height into dwarf, semi-dwarf and tall plants. In M_4 the dwarf types were bushy and easily identifiable, the tall types were semi-spreading with few primary branches, while the semi-dwarf mutants segregated into dwarf, semi-dwarf and tall types. Crosses between dwarf and tall types produced semi-dwarf F_1 plants. The F_2 segregated for dwarf, semi-dwarf and tall types in the ratio 1:2:1, confirming that semi-dwarfism was determined by a single, partially dominant gene.

The determinate growth habit has never been observed in the germplasm collection of chickpea (*Cicer arietinum* L.) at ICRISAT, India. Therefore, Van Rheenen *et al.* (1994) investigated the possibility of inducing this trait. A plant with determinate growth habit was recovered in the M_2 after 15 Krad seed treatment. The mutant plant flowered profusely, but failed to form pods, either with natural or hand pollination. The plant was morphologically determinate, had a bushy growth habit and a reduced number of leaflets per leaf as compared with the ICCV6 parent. The F_1 plants appeared to be normal and non-determinate, suggesting that determinate growth is recessive. The F_2 produced 81 determinate and 379 indeterminate plants, supporting the hypothesis of digenic epistatic inheritance.

Mutants with improved nitrate use efficiency are very important in agricultural production. Ichii *et al.* (1993) obtained mutants with low nitrate reductase (NR) activity, selected from seedings expressing nitrogen deficiency symptoms in rice (*Oryza sativa* L.). Among the 48 000 M_2 seedlings mutagenized with gamma-rays (299 Gy) in a nitrogen-deficient solution with nitrate as the sole source of nitrogen, 77 plants were identified as variants with low NR activity and/or low nitrate uptake. Most of these plants were sterile, but 11 of them produced M_3 seeds. In M_3, two lines were identified as mutants with low NR activity.

A short thick fruit mutant of *Thrichosanthes anguina* L. (a vegetable) was induced by 30 Krad treatment (Datta, 1994). The mutant was isolated in the M_2 from the green–white-striped variety and it differed from the mother line in fruit shape and size. The genetic analysis revealed that the mutation of fruit characteristics was monogenic and recessive. In Brazil, Tulmann Neto and Alberini (1989) released a bushy bean mutant of 'Carioca Arbustivo precoce 1070' (CAP-1070), induced by gamma-rays.

Mutation breeding for the induction of morphophysiological changes is therefore highly recommended, especially when (i) the desired trait is not available in natural germplasm, and (ii) a well-adapted and outstanding cultivar requires few improvements of defective characters. Most induced changes of these types are easily detectable.

Quality

Nishio and Iida (1992) obtained rice (*Oryza sativa* L.) mutants with low allergenic protein content. The main allergen, a 16-kDa globulin, which is heat stable and resistant to degradation by proteolytic enzymes in the human body, has been identified in rice. This protein causes atopic dermatitis from rice consumption, especially among children in Japan, and it has raised many health concerns. Rice mutants with altered composition of seed protein have been obtained by 200 Gy gamma-rays (Co^{60}) irradiation and 0.2% EMS treatment for 5 h (Kumamaru *et al.*, 1988). Four independent mutant lines containing low levels of the 16-kDa allergenic protein were obtained. Two mutant lines, 85KG-4 (from cv. Koshihikari) and 86RG-18 (from cv. Reimei), contained low levels of the 16-kDa polypeptide and a high level of a 57-kDa polypeptide. The amount of the 16-kDa protein in the two mutants was about half that of the original cultivars. From the two mutants homozygous lines were developed, showing normal growth and seed set. The other two mutant lines, 87KG-970 and 89WPKE-149, both from cv. Koshihikari, showed traces of 16-kDa and 26 kDa polypeptides and contained a high level of a 13 kDa polypeptide.

Oda *et al.* (1992) obtained a bread wheat mutant with low amylose content induced by ethyl methanesulphonate. In *Triticum* species, no mutants with 'waxy' endosperm have been reported except for one in *T. monococcum* with glutinous, 'waxy' endosperm starch (Kanzaki and Noda, 1988).

Soybean (*Glycine max* (L.) Merril) seeds contain three lipoxygenase isozymes (L-1, L-2, L-3) which are responsible for the production of grassy beany flavour, and hamper wide utilization of soybean products (Palmer and Kilen, 1987). Mutants lacking L-1 were reported by Hildebrand and Hymowitz (1981, 1982), those lacking L-2 by Kitamura and Kikuchi (1985) and Davies and Nielsen (1986), and L-3 lacking mutants by Kitamura *et al.* (1983). Two types of double mutants which lack both L-1 and L-3 or L-2 and L-3 were obtained by Kitamura and Kikuchi (1985); however, neither double mutant seeds lacking both L-1 and L-2 nor triple mutant seeds lacking all the isozymes have been identified, presumably because of the close linkage between the *Lx1–lx1* and *Lx2–lx2* loci. However, Hajita *et al.* (1991) induced a mutant line lacking all the three lipoxygenase isozymes by gamma-irradiation. This mutant went through two generation cycles without displaying any physiological abnormality, and it was concluded that the induced trait was a truly inherited one.

Nowadays, quality deserves much more attention than in the past. Mutation breeding is regarded as a valuable tool to tailor cultivars for changed food requirements, for feed and for industrial markets.

Oil Content

In sunflower, dry seeds of lines HA-234 (maintainer line of CMS-234) and RHA-274 (male parent of BSH-1 sunflower hybrid) were treated with 0.1%, 0.2%, 0.3%, and 0.4% aqueous solutions of EMS for 8 h, and with 5 and 10 Krad (Co^{60}) gamma-rays (Giriraj *et al.*, 1990). The mutagenic treatments significantly

increased variation for days-to-flowering, test weight and oil content in M_2 and M_3 generations. Early and late-flowering mutants were also isolated from these lines. EMS was more effective than gamma-rays for widening the range for test weight. The induced variation for oil content thus provided the possibility to select for high oil mutants.

In Canada, an EMS-induced mutant for low-linolenic-acid in McGregor flax (*Linum usitatissimum* L.) after seed treatment was reported by Rowland (1991). The parent cv. McGregor has a linolenic acid content of 38–59%. The screening for linolenic acid mutants was carried out in the M_2, M_3, and M_4 generations, using the half-seed technique. A stable mutant for low-linolenic acid (2%) was found in the M_4. The low–linolenic–acid trait is controlled by recessive alleles at two independent loci, apparently the result of a rare double mutation. Two M_4 lines E1747-9-1-5 and E1747-9-7-5 were apparently homozygous for low linolenic acid. The stability of these two lines was confirmed in the M_5 generation.

In Australia, Green (1986) and Green and Marshall (1984) described a mutant genotype of flax (*Linum usitatissimum* L.) with 1.6% linoleic acid in seed oil, well below the 40.1% found in the parental cv. Glenelg. The low–linoleic–acid F_2 plants arose from a cross between two EMS-induced mutant lines of 'Glenelg' with lowered linoleic acid levels. Rowland *et al.* (1995) applied chemical mutagenesis and biotechnology to modify linseed oil composition and isolated several mutants. The mutant E1747 had 2% linolenic acid. The mutant E67 had palmitic acid levels of approximately 28%, which is three to four times higher that in the parent 'McGregor' and three times higher than any previously reported palmitic acid content in *L. usitatissimum.* Ntiamoah (1993) showed that the high palmitic acid level of E67 was controlled by a single partially dominant gene. Finally, the mutant E1929 had oleic acid levels twice that of 'McGregor'.

In rapeseed, several mutants with reduced levels of polyunsaturated fatty acids and increased level of oleic acid were obtained after seed treatment with EMS (Auld *et al.*, 1992). These results are of interest because the existing canola cultivars produce oils with about 55–65% oleic acid, 14–18% linoleic acid, and 8–12% linolenic acid. The relatively high levels of polyunsaturated fatty acids limit the use of canola oil for cooking unless it is hydrogenated.

Rahaman *et al.* (1994) obtained a mutant line of soybean (M23) with a two-fold increase in seed oil content of oleic acid as compared with the original cv. Bay. By reciprocal crossing of the mutant with the parent cv. Bay, no maternal or cytoplasmic effects were observed for oleic acid content. The F_1 seeds and F_1 plants were significantly different from the parents and from the midparent value, suggesting partial dominance of oleic acid content in the crosses. The oleic acid content segregated in the F_2 seeds and F_2 plants in a trimodal pattern with normal, intermediate and high classes, fitting the 1:2:1 ratio. The seed of a backcross between M23 and F_1 segregated into intermediate and high classes in a 1:1 ratio. These results suggested that the oleic acid content is controlled by two partially dominant alleles at a single locus.

In soybean, Takagi *et al.* (1989) also reported a high linolenic acid mutant induced by X-ray irradiation. Soybean seeds cv. Bay were irradiated with X-rays (25 Krad) and the M_2 progenies were screened for changes in fatty acid composition of seed oil. X-rays remarkably increased genetic variation for fatty acid composition of the oil. A mutant was identified among 2006 M_2 plants in which linolenic acid accounted for 18.4% of total oil content, as compared with 9.4% in cv. Bay. The M_3 generation of the mutant had more than twice the linolenic acid content compared with the original variety.

Heterotic Effects

Mutagenesis can be an additional tool to alter and improve the heterotic capacity of the parents that have already gone into the production of hybrids, by inducing disease resistance without losing the fertility restorer genes. Mutations were induced by treating dry seeds of the promising inbred line K560-230 of pearl millet (*Pennisetum americanum* L.) with EMS and gamma-rays (Ravat and Tyagi, 1989). The extent of heterosis in the mutant lines with respect to yield and its components was tested for two consecutive years in a line × tester cross and in a set of crosses involving 20 mutant lines (derivatives of K560-230) which had less than 10% incidence of ergot under artificial inoculation as compared to 50% incidence in the control. Several mutant lines showed higher heterosis than their parent for the majority of studied traits. Heterosis of mutants for grain yield was directly related to 1000-grain weight and to grain yield per plant; in some crosses heterosis was also recorded for the number of productive tillers, seedling vigour and plant height.

In spring barley, a high degree of heterosis was observed in F_1 of mutant crosses. The heterosis reached 20% over the control variety in 37–48% of cross combinations analysed, depending on the character. Significant heterosis was also observed in F_1 from crosses of mutants with their parent varieties (Maluszynski *et al.*, 1989). Maluszynski *et al.* (1995) suggested a breeding scheme which includes the following steps: developing stable mutants, screening for heterosis in the F_1 of mutant crosses, production of doubled haploids from heterotic F_1, screening for 'F_1 performing' doubled haploids, and agronomic evaluation of selected doubled haploid lines.

Disease Resistance

In order to increase the genetic variation for resistance to bacterial leaf blight (BB) caused by *Xanthomonas campestris* pv. *oryzae* in rice, Taura *et al.* (1986) treated fertilized egg cells in the single-cell stage of embryogenesis of the variety 'Taichung Native 1' with methyl nitroso urea (MNU). Two rice mutants resistant to *Xanthomonas* were obtained. Two M_2 plant progenies out of 2739 inoculated at seedling stage with pathogen race 5 (isolate PXO 112), segregated for disease

resistance. Both mutant lines were tested in M_3 generation and their resistance was confirmed. When the two mutants were crossed with 'IR 24' in order to assess the inheritance of resistance, the reaction of F_1 hybrids and F_2 population suggested that both mutants had a single recessive gene for BB resistance.

In sesame, *Sesamum indicum* L., gamma-ray-induced field tolerance to *Phytophthora* blight was reported by Pathirana (1992). Seeds of three genotypes (MI.3, Australian introduction, Kekirawa local) were irradiated with (Co^{60}) gamma-rays (100–750 Gy). Seeds of the first five capsules formed in the M_1 plants were bulked bearing separate varieties and treatments and sown in a field with high disease incidence (total population 28 000 plants). The best 21 lines with the highest survival and seed production were identified in M_3 and M_4 progeny rows of selected M_2 single plants, and tested in replicated field trials with the recommended cultivar MI.3 as a control. Eight selections gave significantly higher seed yield than MI.3.

A mutation breeding experiment for resistance to blackspot bruise and low-temperature sweetening in potato cultivar Lemhi Russet was carried out by Love *et al.* (1993). Following irradiation with gamma-rays from a Co^{60} source (2.5, 3.0, 3.5 and 4.0 Krad), more than 2000 tuber eye pieces were planted and individually evaluated for blackspot bruise while selection was continued for five clonal propagation cycles. Selection for low-temperature sweetening resistance began in M_3 and continued for three cycles. Ten clones were selected, eight with a significantly higher blackspot bruise resistance, and two with an increased resistance to low-temperature sweetening.

Sonnino *et al.* (1991) developed a method of *in-vitro* mutation breeding for resistance to *Phytophthora infestans*, the causal agent of late blight. Potato micronodes were irradiated *in vitro* (50 Gy), micropropagated and screened with the culture filtrate of the fungus. After two cycles of selection, 5.6% of the micronodes survived (Crinó *et al.*, 1990). Five clones obtained from micronodes insensitive to the culture filtrate showed some resistance to the fungal infection (Lai *et al.*, 1993).

Mutants for tolerance or disease resistance are not as numerous in the literature as the mutants reported for other useful traits. This can be explained by the fact that disease resistance is mostly under the control of dominant genes, and the rate for the occurrence of a dominant mutation is relatively low. Monogenic resistance is often race-specific and is usually easily overcome by the evolution of the pathogen population. Therefore, in order to produce long-lasting resistance, it is always advisable to pyramid more than one resistance gene in the same variety.

Non-specific resistances are controlled by the interaction of several genes. The probability of inducing mutations is therefore higher than for major genes, but the phenotypical effects of mutated minor genes are not easily perceivable. As a consequence, selection is rather difficult. In this regard, the approach used by Lai *et al.*, 1993, who studied the behaviour of potato mutants partially resistant to *Phytophthora infestans* during different phases of pathogenesis (infection, tissue invasion, sporulation), seems very promising.

Future Outlook

Mutagenesis as a Tool to Generate Desired Variation

On the basis of the results achieved during the past three decades, both physical and chemical mutagens will continue to be used in order to increase the amount of genetic variation available for crop improvement.

In seed-propagated crop plants, several improved varieties have been developed by direct multiplication of induced mutants for many plant species and these are being used in agricultural and horticultural production. There is clear evidence that induced mutants constitute valuable germplasm for cross-breeding in addition to their direct use as new varieties. There is always an increasing number of plant breeders who are now using induced mutants for cross-breeding, and molecular biologists regard them as a tool to study gene functions (Rahaman *et al.*, 1994; Nishio and Iida, 1992).

In vegetatively propagated plants which are highly heterozygous, mutation breeding is used in addition to sexual breeding for the genetic improvement of specific traits in otherwise well-performing cultivars. Furthermore, mutation breeding combined with *in-vitro* culture is considered as an efficient approach for enhancing the induction and accelerating the recovery of somatic mutations. The advantage of using *in-vitro* mutagenesis is that a large population may be subjected to screening and selection.

Use of Induced Mutants in Analytical Studies

Induced mutants represent unique tools for analysing single gene differences. A new mutant allele, described by William *et al.* (1984), has been recovered following seed treatment of *Lupinus mutabilis* with EMS. The homozygous condition of the mutant reduces the alkaloid seed content from 2.0% to 0.2–0.3%, and produces plants which are organoleptically 'sweet'. As previously stated, induced mutants have played a vital role in advancing our knowledge on photosynthesis. Leaf rust (*Puccinia recondita*) resistant mutants of wheat have been used (Favret, 1976) for increasing knowledge on host–pathogen interactions. In maize and barley the waxy (*wy*) mutants are being exploited to study the fine structure of higher plants' genes. Biochemical genetic analysis of the nitrate reductase-deficient mutants are providing information on the enzyme components, and may prove useful to analyse the fine structure of these genes (Kleinhofs *et al.*, 1980). Mutants of barley and rice affecting gibberellic acid (GA) synthesis and response to external GA application may prove helpful for understanding not only the pathways of GA biosynthesis, but also the role played by GA in growth and height expression. The short straw mutant genes in barley *erectoid* (*ert*) types are located on 29 loci of the barley genome. Most *erectoid* mutants have a reduced spike density, and some have proved useful in cross-breeding. Reduced plant height mutants of barley and wheat have contributed significantly

to broadening the base of genetic variation for this important trait. Lundqvist and Lundqvist (1994), on the basis of 144 barley mutants for variation in seed rows of the spike, demonstrated that at least 12 loci (*hex*-v locus and 11 *int* loci) are responsible for the spike structure and lateral florets, for their size, awn development, fertility and kernel development (Maluszynski *et al.*, 1995).

Mutagenesis and Plant Biotechnology

There are several applications of mutagenesis in basic research, particularly in the identification of changes in a single base-pair of DNA. Genes controlling many plant traits are often unknown, which makes gene technology difficult. Induced mutants, when identified and genetically characterized, may allow the isolation of a target gene, and consequently facilitate plant genetic engineering. Induced mutants for the floral colour and floral morphology in *Petunia* and *Antirrhinum* have been used to study flower morphogenesis and to isolate the gene involved (Schwarz-Sommer *et al.*, 1990). Several molecular biology investigations currently rely on the induction and identification of mutants in model species such as *Arabidopsis* for the construction and subsequent saturation of genetic maps, for the understanding of developmental genetics and the elucidation of biochemical pathways. Once identified and isolated, the genes that encode agronomically important traits can be either introduced directly into crop plants using transformation technology or used as probes to search for similar genes in other species.

Mutagenesis Facilitates Gene Transfer

There are a number of reports of irradiation-induced transfers, mostly in wheat, but also in tobacco and oats. The irradiation-induced transfers are always based on reciprocal translocations between an alien chromosome and a chromosome of the recipient species due to random chromosome breakage and reunion. Such induced transfers can be tolerated only by polyploid species, on account of their genetic duplication, whereas such a situation would be lethal in a diploid species. The transfer of leaf rust resistance from *Aegilops umbellulata* to bread wheat described by Sears (1956) was the first report of the successful use of radiation to insert the critical segment of an alien chromosome into bread wheat. Several transfers of desirable characters have since then been carried out, especially in wheat, through chromosome engineering. Induced mutations for homoeologous pairing regulator genes, and in particular one for the *Ph1* gene of wheat, have been used extensively in recent years to promote homoeologous pairing and recombination between chromosomes of a related wild species and wheat (Ceoloni *et al.*, 1990).

Conclusions

Although, mutation breeding has played a vital role in the past four decades for generating new, valuable cultivars, it is true that the recent advances in plant biotechnology and transformation have eroded and elsewhere diverted much of the attention (and resources) it was receiving in the past. However, mutation breeding, like molecular markers or plant transformation technology, is simply one of the tools which are available to the breeder, and as such it presents advantages and limitations. Furthermore, mutation breeding in the new millennium could well live a new 'youth', thanks to some of the tools offered by molecular biology, and in particular great advantages can be envisaged by the use of molecular markers for selection of mutants or basic research studies (Straus and Ausubel, 1990). Mutagenesis will retain its importance for generating variation in seed-propagated crop plants, and in particular in cereals, at least until the new genetic engineering approaches and transformation technologies can be routinely used. The same applies to the highly heterozygous vegetatively propagated plants for which cross-breeding is not easily applicable (Micke *et al.*, 1987). The most relevant applications of mutagenesis are thus foreseen:

1. To create new alleles for desirable traits, thereby enlarging the genetic variation available to breeders (creation of new sources of semi-dwarfism of importance in rice breeding to replace the gene *sd1*, or in wheat to replace the genes: *Rht1*, *Rht2*, *Rht8*, *Rht9*).
2. To improve important traits in existing well-adapted local varieties which will help preserve germplasm by avoiding the need for introducing improved varieties from different environments.
3. To obtain morphological, biochemical or molecular markers for genetic studies on particular traits.
4. To assist plant biotechnology by generating genetic variation in combination with *in-vitro* culture, including *in-vitro* selection for resistance to ebiotic and abiotic stresses, and by providing a tool for studying gene expression and regulation.
5. To stimulate chromosomal exchanges and to induce translocations leading to the insertion of alien genes from wild species into cultivated plants.
6. To induce mutants at loci controlling homoeologous chromosome pairing which are of great value in chromosome engineering for alien gene introgression.

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15. *In-vitro* Techniques and Mutagenesis for the Improvement of Vegetatively Propagated Plants

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Introduction

Conventional plant breeding is based on genetic variation and selection of the desired genotypes. The availability of genetic diversity and genetic variation is the starting point of any breeding programme. In most crops, sufficient genetic variation is present among land races, cultivars and their wild relatives. In conventional plant breeding programmes this is followed by several years of selection and field evaluation before a desired genotype is released as an improved cultivar. In many sexually propagated crops, e.g., rice, wheat, maize, barley, the genetic variability is usually recombined through hybridization. The desired recombinants are then selected from the segregating populations in the subsequent generations which are tested, multiplied and released as improved cultivars. However, a number of important crops such as banana, plantain, date palm, citrus, potato, sweet potato, pineapple, apple, and pear are propagated from vegetative parts, and are not amenable to improvement in the same manner as sexually propagated plants. Many of these plants are complex polyploids, take several years to flower and fruit, some are self-incompatible. In others, such as banana and plantain, there is little or no seed formation. Often the propagule size is too big and bulky to handle and to grow large populations. Hence, it is not possible to produce sufficiently large populations required to obtain the desired recombinant in a short duration. In these plants, mutation induction offers the possibility to alter a few characters without disrupting the genome while retaining all the other characters of clones.

Plant Breeding and Parasexual Techniques

During the past decade, many *in-vitro* techniques of plant tissue and cell culture have been developed and refined as an aid to conventional plant breeding. The use of *in-vitro* techniques can complement conventional plant improvement in several stages of plant breeding. The plant breeder has available several *in-vitro* techniques (Table 1) which increase efficiency in obtaining variation, selection and multiplication of the desired genotypes. These techniques are particularly valuable in the improvement of plants which are propagated from vegetative parts.

In-vitro culture and recombinant DNA technologies have opened new vistas to complement plant improvement. These techniques are particularly suitable to improve vegetatively propagated plants, and to upgrade a few traits in a well-adapted variety (Ahloowalia, 1995). These include *in-vitro* mutation induction,

S.M. Jain, D.S. Brar, B.S. Ahloowalia (eds.), Somaclonal Variation and Induced Mutations in Crop Improvement, pp. 293–309.

Table 1. Use of parasexual techniques in plant improvement

Technique	Target
Micropropagation	Clonal maintenance, large-scale multiplication, hybrid seed production
Embryo rescue	Wide crosses, widening genetic base, transfer of novel traits
Somatic embryogenesis	Mutation induction, somaclonal variation, selection, artificial seeds
In-vitro fertilization	Use of self-incompatibility in hybrid variety production
Anther/ovary culture	Doubled haploids, inbred lines for hybrids, synthetics, composites
Protoplast fusion	Widening genetic base
Organelle transfer	Cytoplasm manipulation, male sterility
Manipulation of medium composition	Selection for growth, vigour, disease resistance, stress tolerance, herbicide resistance, NPK response, *in-vitro* chemical mutagenesis
Radiation *in vitro* and *in vivo*	Elimination of linkage drag in cybrids/hybrids/wide crosses, induction of chromosomal exchanges
DNA probes	Disease detection, sexing, trait selection, QTL maps
Recombinant DNA	Transgenesis, gene location, insertion/deletion/mapping

embryo rescue, anther and ovary culture, protoplast fusion and the transgenic approach. In many cases *in-vitro* culture and mutation induction techniques can be combined to obtain the desired genotypes. For example, clones can be *in-vitro* cultured, multiplied through micropropagation or regeneration, irradiated, and then multiplied *in vitro* to build up sufficiently large populations for growing in the field for selection. Likewise, cell suspensions, regenerative callus cultures, zygotic and somatic embryos, fused protoplasts and cybrids (somatic hybrids in which cytoplasm of one cell is fused with the nucleus of another cell) can be irradiated to induce variation in addition to that resulting from somaclonal and protoclonal variation. Perhaps it would also be possible to mutate inserted genes in transgenic plants to obtain new alleles. The mutated genes or their altered sequences could then be transferred through either gene insertion or conventional back-crossing. In addition, the linkage drag of unwanted genes in cybrids can be eliminated by irradiation of either protoplasts or fusion products. In many seed-propagated crops, e.g. pepper (*Capsicum* spp.), haploid production from anther culture, followed by the production of doubled haploids can be obtained routinely (Mityko *et al.*, 1996). Irradiation of parental plants before or at anthesis, or even of microspores, can be used to induce variation.

Mutagenesis in Vegetatively Propagated Plants

Most sexually propagated plants are amenable to improvement through conventional hybridization and selection. However, in many vegetatively propagated plants, e.g., banana, plantain, cassava, potato, sweet potato, yams and several ornamentals, sexual recombinants cannot be obtained in the same manner as in the seed-propagated crops. These crops are conventionally propagated from bulbs,

corms, tubers and stem cuttings. Many of these plants either do not produce seed or their seed progeny are highly heterogeneous. In some of them, fertilization does not result in seed production (e.g., in banana and plantain). In others, where interspecific hybridization results in only a few seeds (e.g., plantain × banana crosses) or results in aborted endosperm (e.g., between the cultivated sweet potato, *Ipomoea batatas* and its wild relatives *I. triloba* × *I. lacunosa*) (Kobyashi *et al.*, 1994), embryo rescue can be used to obtain hybrids. In wide crosses, where seed set is extremely low, *in-vitro* culture of embryos and irradiation can be used to widen genetic variation and to obtain structural changes (e.g., translocations) between chromosomes of different genomes. Even when crossing between the cultivated clones and the wild types is successful (e.g. *Musa sapiantum* × *M. acuminata* or *M. balbisiana*) the embryos abort and the number of seeds recovered is small. *In-vitro* culture of such hybrids and irradiation can be used to generate variation. In some wide crosses, the seed progeny combines too many unwanted characters, resulting in undesired genotype. The inactivation of nuclei of cells or protoplasts with high doses of irradiation for producing cybrids has also been used to produce new and novel gene combinations, for example in case of rape seed and radish protoplast fusion-derived plants. Often the conventional propagule such as a stem cutting, corm or tuber is large, and irradiation of large numbers is not convenient. Unlike seed-propagated plants, there are severe limitations on the population size which can be grown for selection and evaluation of clones. For example, in crops such as sugarcane and potato, which are complex polyploids, the progeny is highly heterogeneous with an extremely low frequency of the desired recombinants. In such crops, the initial seed-derived populations required for preliminary selection can be as high as one million seedlings.

Mutation induction and selection of desired traits in combination with *in-vitro* techniques thus offers several advantages over the conventional methods, particularly when the objective is to change one or two characters in an otherwise well-adapted and high-yielding clone. *In-vitro* techniques allow mutagenic treatment of large numbers and multiplication of the selected genotypes in a small space and short duration under disease-free situations, with a minimal risk of loss of variants through accidents during propagation.

Mutagenesis in Ornamental Plants and Fruit Trees

Before the development of *in-vitro* techniques, radiation-induced mutations resulted in an array of new varieties in many ornamentals, such as *Achimenes*, chrysanthemum, carnation, roses and streptocarpus (Broertjes, 1977). Many of these mutants were obtained by irradiating rooted stem cuttings, detached leaves, and dormant plants. The altered flower colour and flower shape, growth habit (dwarf or trailing) and any other novel phenotype of commercial value was immediately recognized. Even the chimaeras (e.g., variegated foliage plants and white and red bract bougainvillea) which normally would be of limited value in crop plants such as potato and banana, became popular among growers. The FAO/IAEA database of induced mutants in vegetatively propagated plants shows that of the 465

Table 2. Mutant varieties released in ornamentals

Plant	No. of varieties
Chrysanthemum	187
Alstroemeria	35
Dahlia	34
Streptocarpus	30
Rose	27
Begonia	25
Carnation	18
Azalea	15
Bougainvillea	9
Achimenes	8
Others	77
Total	465

After Maluszynski *et al.*, 1992.

mutants released among the vegetatively propagated plants, most have been among the floricultural plants (e.g., chrysanthemums, alstroemeria, dahlia, rose, begonia, carnation) and fruit trees (Table 2). Among these are released mutants in chrysanthemum (187), alstroemeria (35), dahlia (34), bougainvillea (9), rose (27), achimenes (8), begonia (25), carnation (18), streptocarpus (30), azalea (15) (Maluszynski *et al.*, 1992). Since the effect of mutation in ornamentals is very visible, selection for changed flower colour, shape or size, does not require elaborate breeding procedures, and almost anything which is novel is marketable. Hence, there has been a high success rate for breeding ornamental plants based on mutation techniques where this technology has become a major tool (Maluszynski *et al.*, 1995).

Very few mutant varieties have been produced in vegetatively propagated fruits. These include the release of changed skin-colour apple mutant in Austria (Brunner and Keppl, 1991), disease-resistant mutant in Japanese pear in Japan (Sanada *et al.*, 1993), spineless mutant of pineapple in the Philippines (Lapade *et al.*, 1995), and 'Novaria', an early-ripening mutant with enhanced flavour in banana in Malaysia (Mak *et al.*, 1996), disease-resistant mutants of banana in Cuba and Costa Rica, and reduced tuber glycoalkaloid content in potato (Love *et al.*, 1996) (Table 3). However, the technology remains unexploited in many other vegetatively propagated crops such as sweet potato, yams, plantain, strawberry and date palm.

In-vitro Techniques for Mutagenesis

In-vitro techniques allow induction of mutations in large numbers of propagules in a small space, and several cycles of subculture can be carried out in a short

Table 3. Induced mutants and their characters in some horticultural crops

Crop	Mutant	Character
Chrysanthemum	Many	Flower colour
Roses	Many	Flower shape, size
Bougainvillea	Many	Bract colour
Potato	–	Skin colour, texture, eye depth, reduced glycoalkoid
Apple	Golden Heidegg	Changed skin colour
Japanese pear	Golden Nijisseiki	Black spot disease resistance
Orange	Hongju 418, Hongju 420	Seedless
Lemon	–	Seedless
Grapefruit	Rio Red	Deep red flesh and juice colour
Banana	Novaria	Enhanced flavour
Banana	–	Disease-resistant to sigatoka
Pineapple	–	Spineless mutant
Blackberry	–	Thornless
Peach	–	Early maturity

duration to separate the mutated sectors from non-mutated ones, and to increase the population to sufficiently large numbers for selection. In addition, there is no loss of the mutants, because micropropagules are subcultured under insect- and disease-free conditions. Culture of apical meristems, axillary buds, micro-cuttings and micro-plants, regenerative and embryogenic calli and cell suspensions, all allow mutagenic treatment of millions of cells with physical or chemical mutagens. Following mutagen treatment, *in-vitro*-cultured tissues are chimaeras, composed of mutated and non-mutated cells and a mixture of several mutations. It is therefore necessary to separate various sectors through subculture into homo-histonts. Because only a few cells, even in an organized apical meristem, are involved in giving rise to a bud and shoot each time it is propagated, repeated *in-vitro* propagation of irradiated tissues rapidly separates such sectors. Usually, three or four propagations after irradiation are considered sufficient to achieve this separation, often referred to as 'dissolving a chimaera to obtain homo-histonts'.

It is common practice to include the chemical mutagens in the media, and determine the LD_{50} by varying the doses of mutagens. Likewise, in irradiation experiments, it is essential to determine LD_{50} by giving a series of irradiation doses and comparing the survival of the subsequently propagated subcultures with that of the control (unirradiated) material. In general, *in-vitro*-cultured plant cells and tissues require lower doses of irradiation than seeds to induce mutations. Although species and genotypes may differ in LD_{50}, based on survival of M_1 V_2 and M_1 V_3 propagules, doses of 20 Gy gamma-rays were found to be optimal for tissue cultures of potato, chrysanthemum, streptocarpus, and date palm; LD_{50} being about 25 Gy (unpublished data).

A range of plant species can now be micropropagated or regenerated through organogenesis and somatic embryogenesis. These include vegetables – potato, sweet potato, garlic, ginger; ornamentals – chrysanthemum, streptocarpus, roses,

oleander, cyclamen, gladiolus, iris; fruits – apple, banana, citrus, grape, mulberry, strawberry. The irradiation of cultures, which are capable of producing somatic embryos from single cells, is particularly important since one can obtain solid mutants quickly and in large numbers. Increasing success in obtaining somatic embryogenesis in diverse species makes irradiation of *in-vitro*-cultured plant material a very powerful tool for the improvement of vegetatively propagated plants.

Mutagenesis and Micropropagation

In most vegetatively propagated plants, micropropagation is sufficient for irradiation of *in-vitro* cultures, and the techniques of micropropagation are well developed for most of them. It is recommended that for the irradiation of microporpagules, e.g., micro-tubers, micro-plants, simple media such as MS (Murashige and Skoog, 1962), preferably without growth regulators, should be used to initiate and establish cultures. A half-strength MS medium (Ahloowalia, 1975) containing 3% sucrose, was found to be adequate for explant initiation, maintenance and growth of *in-vitro*-cultured plants of chrysanthemum, carnation, ginger, potato, pineapple and sugarcane. In banana and plantain, use of growth regulators such as kinetin (KIN) and indole butyric acid (IBA) is essential to culture explants; this leads to a certain amount of somaclonal variation, depending upon the genotype. In such cases, explants should be irradiated immediately after culture, and then multiplied on media containing growth regulators along with the unirradiated controls. It is essential that the explants are transferred to fresh medium immediately after irradiation to avoid toxic compounds in the medium. The subculture of irradiated explants (M_1) for two to four times (V_1 through V_4) results in the separation of the chimaeral tissues, whether originating from mutagenesis or somaclonal variation. In most cases, conventional propagation from cuttings or tubers is required before selection of variants. This eliminates physiological effects of tissue culture which may simulate somaclonal variation. For example, *in-vitro*-grown potato plants were irradiated, and multiplied in large numbers from internodal stem-cuttings and micro-tubers 2–10 mm diameter were obtained on half-MS medium without any growth regulators (Ahloowalia, 1990). The successive bud culture on the same medium resulted in separation of the mutated from the non-mutated sectors. Likewise in chrysanthemum (Ahloowalia, 1992), irradiation of *in-vitro* plants with 20 Gy gamma-rays and their subsequent propagation produced several interesting solid variants. Similar results were obtained in carnation and streptocarpus following *in-vitro* irradiation with 20 Gy (Ahloowalia, unpublished).

Many fruit trees (date palm, banana, plantain, apple, pear) and bulky materials (potato tubers, sweet potato, yams, tulip and daffodil bulbs) can now be irradiated *in vitro* as micro-cultured plants, thus allowing mutagenic treatment of large populations, a prerequisite for a successful breeding programme. Thus, the combination of *in-vitro* culture and mutation techniques is simple, fast and efficient to improve horticultural crops. For example, microcultured plants of chrysanthemum, potato, and streptocarpus were irradiated with 20 Gy gamma-rays, and sub-

cultured four times from single nodal cuttings (Ahloowalia, unpublished). In each plant, several useful mutants were recovered among the populations thus produced. In potato, micropropagated plantlets were irradiated and micropropagated three times and then in the M_1 V_3 allowed to produce micro-tubers *in vitro*, which were then planted in soil for selection. Among the M_1 V_4 propagules, variants with shallow eye, changed tuber shape, skin colour and tuber size were recovered which propagated true to type in the three subsequent propagations (Ahloowalia, 1990). Similarly, in chrysanthemum, even among a small population of 105 *in-vitro*-cultured M_1 V_3 plants, which were obtained by irradiating only two *in-vitro*-grown plantlets, 20 mutants with changed flower shape, colour or floret size were obtained. Of these, 15 mutants were solid, uniform and stable in the subsequent three propagations (Ahloowalia, 1992). Likewise, *in-vitro* irradiation of streptocarpus gave mutants with changed flower colour and of carnation with changed flower shape, colour and leaf size (Ahloowalia, unpublished). Changed flower colour mutants have also been reported in *Dianthus caryophyllus* cv. 'Mystere' following irradiation of *in-vitro* nodal cuttings with X-rays (Cassells *et al.*, 1993).

Mutagenesis and Plant Regeneration

Plants may be regenerated from irradiated callus and suspension cultures through organogenesis or somatic embryos or after irradiation of donor plants and explants, such as meristems, immature seeds and zygotic embryos. In many plants, organogenesis and somatic embryogenesis are preceded by a callus phase, which in addition to the induced mutations, may lead to somaclonal variation, thus providing additional variation for selection. Irradiation of haploid tissues, such as microspores and ovules, and subsequent plant regeneration is also promising. In many crops, it is now possible to obtain haploid plants from either anther or microspore culture and through wide-cross pollination followed by embryo rescue, e.g., wheat × maize, wheat × sorghum, wheat × ryegrass. Induction of mutations in haploid plants, haploid callus cultures and subsequent plant regeneration from them allows expression of the recessive mutations, first in the hemizygous and then in the homozygous state in the doubled haploids. Since population size is important for selection, a combination of whole plant irradiation at the appropriate stage of flowering, followed by plant regeneration from anther/microspore culture provides an efficient method to recover the desired mutants in a homozygous condition in a short duration.

Mutagenesis and Somatic Embryogenesis

Of all the tissue culture methods, somatic embryogenesis is perhaps the most useful in mutagenesis. Somatic embryos usually originate from single or few cells. As a result, non-chimaeric mutants can be obtained from the irradiated mother explants rapidly. The irradiation of callus cultures which are capable of producing somatic embryos of single cell origin is of major interest since one can

obtain homo-histonts quickly and in large numbers. When secondary somatic embryogenesis occurs, the chance of obtaining solid mutants is even better than primary somatic embryos. The production of primary and secondary somatic embryos in large numbers is particularly useful in inducing solid mutants after a short subculture following irradiation.

Recent successes in obtaining somatic embryogenesis in many species make the combination of somatic embryogenesis and irradiation a very powerful tool in the improvement of floricultural plants, such as *Alstroemeria*, in which the whole cycle from culture initiation to rooted plants can be completed in 28 weeks (Schaik *et al.*, 1996). An increasing number of plants can be micropropagated or regenerated through organogenesis and/or somatic embryogenesis. Plant regeneration through organogenesis has been shown in roses, *Rosa hybrida*, *Rosa chinensis minima* (Hsia and Korban, 1996); mulberry, *Morus alba* (Susheelamma *et al.*, 1996); papaya, *Carica papaya* (Drew, 1992); date palm, *Phoenix dactylifera* (Tisserat, 1984); and taro, *Colocasia esculenta* (Sabapathy and Nair, 1995). Somatic embryogenesis has been demonstrated in vegetables such as *Allium fistulosum* L. (Kim and Soh, 1996); potato, *Solanum tuberosum* (Garcia and Martinez, 1995); sweet potato, *Ipomoea batatas* (Bieniek *et al.*, 1995; Sonnino and Mini, 1993); ornamentals, such as roses, *Rosa hybrida* and *Rosa chinensis minima* (Hsia and Korban, 1996); oleander, *Nerium oleander* L. (Santos *et al.*, 1994); cyclamen, *Cyclamen persicum* (Kreuger *et al.*, 1995); gladiolus, *Gladiolus hort.* (Stefaniak, 1994); and fruit trees, such as banana, *Musa* sp. (Krikorian and Cronauer, 1984); grape, *Vitis vinifera* (Hebert-Soule *et al.*, 1995); mulberry (Susheelamma *et al.*, 1996) and date palm, *Phoenix dactylifera* (Cheikh *et al.*, 1989). The irradiation of callus cultures which are capable of somatic embryogenesis and organogenesis can be used to obtain mutants quickly and in large numbers.

Somaclonal Variation and Mutations

Somaclonal variation may provide additional variation to that induced through mutagenesis. Tissue-culture-generated variation such as somaclonal and protoclonal variation may also be useful in the improvement of horticultural plants. As early as 1975, somaclonal variation had been reported among the regenerated plants of ryegrass which included albinos (Ahloowalia, 1975) and those with altered leaf shape and plant vigour. Meiotic studies showed that this variation was associated with chromosomal variation which involved numerical changes in the whole genome, e.g., polyploidy and aneuploidy, and structural alterations such as deletions, translocations and inversions (Ahloowalia, 1976, 1983). Such changes are usually observed among plants which have been treated with high doses of X-rays. In tomato, 13 different single gene mutations were reported among the 230 regenerated plants (Evans and Sharp, 1983). Such mutations are normally obtained among plants irradiated with gamma-rays, neutrons or treated with chemicals such as ethyl methane sulphonate. The observed variation among regenerated plants and those obtained following mutagenic treatment suggests that somaclonal

variation results from a mutagenic process, although the frequency and spectrum of variation is quite different among the somaclones (Ahloowalia, 1986).

In-vitro Selection of Mutations

Selection of the desired genotypes is a critical step in plant breeding. Conventional selection procedures are based on growing large plant populations in a relatively uniform environment – uniform soil fertility, water regime, and an even application of insecticides, fungicides, and inocula. To minimize the effect of uneven environment on the expression of the traits under selection, the material is planted in replicated trials, and appropriate statistical procedures are used to partition the effect of environment from that of the genotype. Hence, the conventional procedures of selection require growing thousands of plants over several seasons and locations.

It has been suggested that *in-vitro* culture techniques may allow screening of large populations of cells (Maliga, 1984) and regenerated plants in a small space and in a much more controlled environment than in the conventional field trials. However, this is possible only when a trait is amenable to *in-vitro* selection, and is expressed and transmitted in the regenerated plants and their progenies. The high efficiency of *in-vitro* selection systems is based on the fact that it is possible to grow millions of cells in a Petri dish or in a flask and achieve rapid multiplication of cell populations on defined media. Following mutation induction, the regenerated plants can be selected *in vitro* by exposing regenerative cells and tissues to stress conditions, and the preselected plants can then be tested in the field. Alternatively, the regenerated plants can be grown in the field, and subjected to selection. The term 'variant' is used to define a new phenotype. Only genetic or molecular evidence can establish its status as a mutant. In many cases the *in-vitro* selected variants were found to be mutants, and showed simple Mendelian inheritance.

Traits Amenable to In-vitro *Selection*

The characters which may be amenable to *in-vitro* selection include: tolerance to heat, cold and freezing injury, day-length, salt tolerance, fungal and bacterial resistance (by co-culture of host with toxins or pathogens), drought tolerance, resistance to herbicides, antibiotics, tolerance to the toxicity of metals (e.g., aluminium, copper, zinc, lead), and low inputs of NPK, resistance to root nematodes (by co-culture of cysts and plant roots), improved symbiosis for nitrogen fixation (by co-culture of bacteria or mycorrhizae with plant roots or whole plants).

The possibility to select some of the above-mentioned traits *in vitro* has been considered by many researchers. As early as 1959, *in-vitro* selection was reported for temperature variation in snapdragon (*Antirrhinum*) cell suspension cultures (Melchers and Bergmann, 1959). The use of cell culture for mutant selection, the ease of screening large cell populations, and the convenience of mutant induction

and selection using haploid cell populations was also pointed out. During the 1970s several reports were published on the development of such methods (Binding *et al.*, 1970; Carlson, 1973). Many *in-vitro* selected variants were investigated for their value in plant improvement. Genotypes tolerant to salinity were obtained in alfalfa, *Medicago sativa* (Winicov, 1991), to drought and soil acidity in sorghum, *Sorghum bicolor* (Duncan *et al.*, 1995), with increased proline and frost tolerance in winter wheat (Dörffling *et al.*, 1993), sodium chloride tolerance in tobacco (Dix and Street, 1975; Nabors *et al.*, 1980), salt and drought tolerance in proso millet (*Panicum miliaceum* L.) (Nabors, 1983), salt tolerance in rice (Vajrabhaya *et al.*, 1989) and Indian mustard (Kirti *et al.*, 1991; Jain *et al.*, 1991), and salinity tolerance in sugarcane in Cuba (Maribona, 1994). The use of haploids for *in-vitro* selection for tolerance to salinity was demonstrated in datura (Tyagi *et al.*, 1981). The possibility to select for resistance to fungal pathogens, such as *Helminthosporium* in maize (Gegenbach *et al.*, 1977), *Phoma lingam* in *Brassica napus* (Sacristan, 1982), *Phytophthora infestans* in potato (Behnke, 1979, 1980), *Helminothosporium sacchari* in sugarcane (Heinz *et al.*, 1977), to bacterial pathogen *Pseudomonas syringae* (Carlson, 1973), to herbicide, e.g. picloram (Chaleff, 1983), chlorosulfuron and sulfometuron methyl (Chaleff and Ray, 1984), paraquat (Miller and Hughes, 1980) in tobacco, and glyphosate in *Petunia hybrida* (Shah *et al.*, 1986) and for changed metabolism in tobacco (Muller, 1983) has also been reported. However, few if any of these variants have proven to be of value in the release of improved varieties. In most cases, either the variants obtained were not investigated for the transmission of the selected trait or were not found to be stable. On the other hand, resistance to herbicides and antibiotics, e.g., kanamycin, chloramphenicol, streptomycin, lincomycin, has been used to develop selectable markers in studies on somatic cell fusion and to detect gene insertion in transgenic plants. Resistance to antibiotics, which in many cases is non-nuclear and located either in the chloroplasts or mitochondria, was selected *in vitro* by adding antibiotics to the media, and is maternally inherited.

In-vitro methods can also be used for the selection of inducible resistance in plants. It is now known that plants produce novel proteins when challenged by a pathogen or by stress. Some of these proteins, especially those which belong to the chitinase and glucanase families, have shown a broad-spectrum fungicidal effect. One of these, a 24 kDa anti-*Phytophthora* protein has been isolated, and the corresponding genes cloned. Likewise, heat-shock proteins are being isolated to identify genes controlling tolerance to high temperature stress.

A number of compounds (enzymes, e.g., peroxidases from horseradish, flavour compounds, drugs, pesticides, and essential oils for industrial use), which are secreted by cells and tissues in the liquid media are also amenable to selection *in vitro.* This concept may also be extended to the selection of cell lines which over-produce secondary metabolites in suspension cultures. Production of compounds in bioreactors from free and immobilized cell suspensions on a large scale is already a commercial proposition.

In-vitro *Selection for Stress Tolerance*

Stress conditions during plant growth reduce crop yield and impair quality. Stress conditions are initiated from extremes of environment, such as drought, flooding, high temperature, freezing, soil salinity, acidity, toxicity caused by high levels of aluminium, lead, copper and zinc. Usually, stress conditions have a cascading effect; for example, high temperature stress may lead to drought and soil salinity. Likewise, high aluminium causes low pH, acid soils and reduces phosphorus availability and uptake.

In-vitro techniques of plant cell and tissue culture allow regeneration, mutation and preselection and multiplication of genotypes in large numbers in a small space, short duration and on a year-round basis. Following mutation induction, the regenerated plants can be preselected *in vitro* by exposing regenerative cells and tissues to stress conditions, and the reselected genotypes can then be selected for the desired characters in the field. Alternatively, the regenerated plants can be grown in the field and subjected to stress conditions.

It has been reported that ABA (abscisic acid) controlling genes may be involved in regulating the genes for stress tolerance to drought in *Arabidopsis thaliana* (Yamaguchi-Shinozaki and Shinozaki, 1993), and freezing tolerance in bromegrass, *Bromus inermis* (Lee and Chen, 1993). Once the genes have been identified, then gene cloning and manipulation becomes the obvious next step for the transfer of such genes to non-resistant types. Such molecular approaches for the selection and engineering of salt-tolerant crops have been suggested (Grover *et al.*, 1993). These include subjecting divergent genotypes, cultivars and land races to sublethal stress and inducing tolerance with abscisic acid in tissue and cell cultures. There is an urgent need to develop similar procedures for *in-vitro* selection of vegetatively propagated plants.

Protocol for In-vitro *Irradiation and Selection*

The following protocol is suggested for handling breeding material in vegetatively propagated plants:

1. Establish *in-vitro* cultures from indexed, virus- and disease-free material. Multiply *in vitro* into large populations.
2. Irradiate cells, tissues, organs meristems, axillary and apical buds, and embryogenic cultures capable of plant regeneration. *In-vitro*-grown plantlets and microtubers may also be used. It is advised to determine LD_{50} by irradiating between 20–30 cultures each with doses ranging between 5 and 60 Gy gamma-rays and determine the optimal dose from the survival of M_1 V_2 propagules. Unirradiated controls must be propagated along with the irradiated material. In several species, doses around 20 Gy gamma-rays have been found to give optimal survival.
3. If possible, use hormone-free media before and after irradiation to avoid physiological effects of culture medium and to minimize somaclonal variation.

4. Irradiate sufficient populations which, after three or four *in-vitro* propagations give about 10 000–20 000 plants for selection. Apply selection pressure in one single sharp dose rather than in step-wise increase. *In-vitro* cultures are known to tolerate stress through adaptation and habituation rather than genetic resistance.
5. Handle M_1 V_4 in batches of 5000 or less as manageable populations for selection. Plant the selected material in the field in batches; if possible retain duplicates of selected clones *in vitro* as micro-propagules. Multiply *in vitro* the selected clones for release.

Other Uses of Mutagenesis

The recombinant DNA technology is now well advanced to insert genes of interest in selected crop varieties. New transgenic varieties of tomato, potato, and squash have already been released (Dale, 1995), and many more will follow in the next few years. The inserted genes in transgenic genotypes could be mutated to induce variation in the transgenes. The new mutated genes or their altered sequences could then be transferred either through gene insertion or conventional back-crossing. Mutations can also be used to delete the selectable markers such as resistance to antibiotics and 'reporter' genes such as GUS which are of no agronomic value but are often retained in the transgenic varieties. As to who shall own the rights on the mutated transgenes in the new genetic background is an open question. Protection of such genes perhaps could not be allowed as a continuation of the patent on the original gene.

The linkage drag of unwanted genes in interspecific sexual hybrids and parasexually generated hybrids from protoplast or cytoplasm fusion (cybrids) can be eliminated by irradiating fusion products. Embryo-rescue has produced many recombinants which could not be obtained otherwise through conventional crossing. The germination of such embryos produces only a few plants and often with unwanted traits. Such embryos could be irradiated *in vitro* to break the linkage of the undesired genes as demonstrated in the classical experiments on wide-crosses between *Aegilops umbellulata* Zhuk. and wheat, *Triticum aestivum* L. em Thell. (Sears, 1993).

Use of Modular Systems in Mutagenesis

The availability of efficient and rapid methods of mutation induction, selection and multiplication is essential to obtain and release mutants. This can be accomplished by using the concept of modular systems which allow batch handling of large populations. Such a modular system of micropropagation based on disposable plastic containers which are then used for growing plants to maturity has been developed. This system can also be used for *in-vitro* irradiation for mutation

induction, preselection, rapid multiplication, and distribution of disease-free propagules. The system consists of 12 culture vessels, with snap-on lids, held in a plastic tray, which allows batch handling of cultures during transfer, culture, hardening, soil transfer of plants and tier-stacking in the growth room. This system can be used both for the rapid multiplication and mutagenesis of vegetatively propagated plants; for example, the subculture of irradiated micro-plants to form micro-tubers and their subsequent soil planting has been used for obtaining several desirable mutants in potato cultivars (Ahloowalia, 1990). The system allows production of potato mini-tubers as a source of seed in large numbers (Ahloowalia, 1994). Similarly, explants of chrysanthemum and carnation were irradiated and multiplied using this system to obtain new floral types.

To understand the molecular basis of induced mutations, one has to look at the structure of the gene and the chemicals which make the gene, and the physical (Briggs and Constantine, 1977) and chemical mutagens (Heslot, 1977) used for inducing mutations (Anonymous, 1977). It is then easily recognized that the mutability of the gene is built into the structure of DNA and its replication. Most of the time, DNA is copied exactly, but infrequently mistakes occur in its replication, resulting in mutations. Such mutations may originate from deletions in the nucleotide sequences in the exons (structural genes), changes in the promoter sequences, deletions of the introns, regulating genes and repressors, which may either cause frame shift mutations or lead to the production of null mutants or modified gene expression.

Conclusions

Plant breeding has resulted in the production of an array of cultivars suited to the specific needs of each eco-climatic region and industry. In doing so, plant breeders have made use of field plot techniques, statistical analysis, and growth in a controlled environment for generation advancement, and thus increased the efficiency of genotype selection. *In-vitro* techniques alone or in combination with mutagenesis offer the plant breeder additional possibilities to enhance variation, hitherto not available in gene pools, to select the desired genotypes more effectively among large populations and multiply the selected genotypes in large numbers as high-quality planting material. Application of radiation or mutagenic chemicals to *in-vitro*-cultured cells, tissues, somatic embryos, protoplast fusion products and embryos rescued from wide crosses, can generate variation, usually not obtained through conventional breeding methods. The combination of mutagenesis with *in-vitro* techniques thus offers an efficient method to improve vegetatively propagated plants. These techniques allow induction of variation, selection and multiplication of the desired genotypes in a much shorter duration and smaller space than the conventional methods.

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16. Mutation Breeding in Cereals and Legumes

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Introduction

During the past more than 50 years, mutation breeding has been successfully utilized for crop improvement, to supplement the efforts made using conventional methods of plant breeding. As early as 1942, the first mutant for disease resistance was reported in barley showing resistance to powdery mildew (Freisleben and Lein, 1942). This encouraged further work on mutation breeding, leading to the release of mutant cultivars in several crops. The FAO/IAEA Mutant Varieties Database contained 1790 accessions in June 1996. Among these varieties, 854 were of cereals and 216 of legumes. The majority of varieties among the cereals came from rice (324), barley (256) and bread wheat (146). Many of these mutant varieties were released during the past 10 years (Maluszynski *et al.*, 1991, 1992, 1995). Although, one may not accept that all the listed mutant cultivars really resulted from induced mutations, there is no doubt about the potential which mutation breeding offers for crop improvement. The attributes which are reported to have been improved by mutation breeding include a wide range of characters, including tolerance to biotic and abiotic stresses, duration of flowering and maturity, and other yield-contributing characters (Micke, 1984, 1988, 1991). Due to recent interest in new biotechnology, induced mutations have also proved useful in the preparation of genetic maps (Schwarzacher, 1994) that will facilitate molecular marker-assisted plant breeding in the future.

Cereals and legumes represent the two most important groups of food crops, so that an improvement in these two groups of crops has been the major concern of plant breeders over the years. In the past, these crops have been improved through several conventional approaches of plant breeding (including introduction, selection and hybridization), using either the available genetic variability or the genetic variability released by recombination of the existing alleles at the available gene loci. However, mutations provide an opportunity to creat hitherto unknown alleles, so that the plant breeder does not remain handicapped due to limited allelic variation at one or more gene loci of interest. This possibility has been exploited in a large measure both in cereals and legumes, as is evident from the list of mutant cultivars released in cereals and legumes (Table 1). In our own laboratory at Meerut, also, we extensively utilized this approach for creating genetic variability in several cereals/millets and legumes, both for academic interest and for application in plant breeding (Gupta and Yashvir, 1976; Yashvir *et al.*, 1975; Birhman and Gupta, 1980; Sharma and Gupta, 1981; Gupta *et al.*, 1994; Gupta, 1998). In this chapter a brief account of history, methods, and achievements of mutation breeding in cereals and legumes is presented.

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Table 1. Number of released varieties developed through mutation breeding in cereals and legumes (MBNL 41, 1994)

	Number of varieties developed		
	Directly	Through crossing	Total*
Cereals			
Avena sativa (oat)	5	10	15 (18)
Hordeum vulgare (barley)	38	201	239 (256)
Oryza sativa (rice)	203	81	284 (324)
Secale cereale (rye)	4	–	4 (4)
Triticum aestivum (bread wheat)	105	34	139 (146)
T. turgidum (durum wheat)	8	17	25 (25)
Zea mays (maize)	9	38	47 (47)
Total	372	381	753 (820)
Legumes			
Arachis hypogea (groundnut)	20	17	37 (38)
Cajanus cajan (pigeonpea)	4	1	5 (5)
Cicer arietinum (chickpea)	7	–	7 (8)
Dolichos lablab (hyacinth bean)	1	–	1 (1)
Glycine max (soybean)	38	3	41 (49)
Lens culinaris (lentil)	1	–	1 (1)
Phaseolus vulgaris (french bean)	15	4	19 (21)
Pisum sativum (pea)	16	14	30 (32)
Trifolium alexandrinum (Egyptian clover)	1	–	1 (1)
T. incarnatum (crimson clover)	1	–	1 (1)
T. pratense (red clover)	1	–	1 (1)
T. subterraneum (subterranean clover)	1	–	1 (1)
Vicia faba (faba bean)	6	2	8 (13)
Vigna angularis (adzuki bean)	1	–	1 (1)
V. mungo (black gram)	1	1	2 (3)
V. radiata (green gram or mungbean)	9	–	9 (12)
V. unguiculata (cowpea)	9	–	9 (9)
Total	132	42	174 (197)

*Figures in parentheses are up-dated values as in June 1996 (supplied by Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture).

History of Mutation Breeding in Cereals and Legumes

The first experiments on X-ray-induced mutations in cereals were conducted as early as 1928, in barley (Stadler, 1928a) and maize (Stadler 1928b). However, these mutations were largely deleterious, so that Stadler considered induced mutations to be a wasteful exercise for plant breeding purposes. Particularly, in a cross-bred crop such as maize, Stadler believed that the naturally occurring genetic diversity was sufficient for selection and hydridization leading to the development of new cultivars. As mentioned earlier, the first efforts to utilize

induced mutations for plant breeding were actually made in barley by Freisleben and Lein (1942, 1943a,b) at Halle in Germany. This work could not be pursued due to World War II (Hoffmann, 1959). Similarly, the first undoubted case of chemical mutagens was discovered during World War II, although the results were published only in 1947 (Auerbach and Robson, 1947). The latter results included the discovery of two chemical mutagens, mustard gas in Scotland and urethane in Germany. Mustard gas belongs to a group of chemical mutagens described as alkylating agents, many of which were later discovered to be mutagenic and superior to X-rays. These were first used in fruitfly (Muller, 1927) and then in barley and maize (Stadler, 1928a,b).

The realization that induced mutations can be used for plant breeding, led to the induction and isolation of mutations for early and late flowering, short straw, and altered spike architecture in barley, wheat and oats. New varieties of rice carrying improved characters due to induced mutations were produced in Asia (Sigurbjörnsson and Micke, 1969, 1974; Wang, 1986). Desirable mutants (having improved characters such as increased yield, earliness, lodging resistance and disease resistance) were also isolated in legume crops, e.g. pea and soybean (Gustafsson, 1947; MacKey, 1956; Anonymous, 1995, 1996a). Efforts were also directed during this period to determine the optimum treatment conditions, and also to find out the possibility of using post- and pre-irradiation treatments leading to desired results. Since mutations were largely random, and had deleterious effects, efforts were also made to discover effective and efficient treatments to induce more specific, directed and economically useful induced mutations in several crops including cereals and legumes (Nilan *et al.*, 1965, 1973).

FAO/IAEA's Activities on Mutation Breeding

In view of the significant role of induced mutations in plant breeding witnessed after World War II, the International Atomic Energy Agency (IAEA), Vienna, (established in 1957) took up the responsibility of encouraging efforts directed towards the use of induced mutations for plant breeding. The Plant Breeding Unit of the IAEA supported the programmes of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (established in 1964). Under this programme, the following activities were included: (i) research and development in mutation breeding techniques; (ii) training of scientists from developing countries in mutation breeding methods, and (iii) service and technical advice for mutagen treatment. As a follow-up action, therefore, in 1969, the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture started organizing training courses for production and use of induced mutations, and also published the first edition of the *Manual on Mutation Breeding*. In view of this, the year 1969 has also been considered as the year when the foundation for mutation breeding as a tool for development of improved cultivars was really laid down (Micke *et al.*, 1990). Some of the desirable traits that were dealt with by induced mutations included resistance to biotic and abiotic stresses, duration of

flowering and maturity, yield components, and improved grain quality in cereals and legumes.

Under the FAO/IAEA Co-ordinated Research Programme (CRP) for 'Improvement of Cereals in Latin America through Mutation Breeding' (Anonymous, 1996b), a number of mutants became available for the improvement of economic traits, such as the following: (i) tolerance to aluminium toxicity (wheat), (ii) increased tolerance to phosphorus deficiency (wheat), (iii) early maturity (rice), (iv) resistance to blast (rice) and *Septoria* (wheat), (v) naked seed (barley), (vi) improved grain quality (rice), (vii) improved leaf shape and semi-dwarfism (rice, barley and wheat). Some of these mutants were already tested in multilocation trials and gave yields higher than those of the leading local varieties. Consequently, in Peru, a mutant barley variety (UNA-La Molina 95) was obtained through irradiation of variety Buena Vista, produced in 1990 through conventional breeding. This new mutant variety has the following three attributes: (i) it matures 3 weeks earlier than the parent variety, enabling it to reach ripening stage when the dry season arrives; (ii) it is shorter, giving it protection against wind and hailstorm; (iii) it produces naked huskless grain with higher protein content, making it easier to cook and feed animals. The success of this variety will be known in 2–3 years, when farmers grow it on many thousands of hectares IAEA, 1996. Based on the results under the Coordinated Research Programme, it is expected that a few new mutant varieties of rice, barley and wheat will be officially released during the next few years in Latin America. Other mutants will be constantly used in conventional breeding programmes.

Among Asian countries, with the assistance of the Department of Technical Cooperation of IAEA, during 1995 nine rice varieties obtained through induced mutations were grown in five provinces of China giving a yield benefit of 263 000 tonnes valued at US$50 million. The area covered by these varieties was 990 000 hectares in 1996 as against 598 000 hectares in 1995. Similarly, in Myanmar the rice mutant '*Shwewartun*' was grown in an area over 800 000 hectares (17% of total rice area) during 1990–93 (see IAEA, 1996).

In Africa, under a FAO/IAEA CRP, improved strains of rice and sorghum were produced in Mali. Initial field tests show increases of 10% in sorghum production and 15% in rice (IAEA, 1996).

Mutagenic Treatments

The most important mutagens used for mutagenic treatments include X-rays, fast neutrons and a variety of chemicals (ethylmethane sulphonate, sodium azide, etc.). The treatments are generally given to seed, seedlings, and gametophytes, although in vegetatively grown crops, bulbs, corms, tubers, scions, cuttings, etc. are also used. However, in recent years, cultured tissues and cultured cells have also become the favourite material for mutagenic treatments. At IAEA, 4892 fast neutron treatments and 13 265 ^{60}Co gamma-ray treatments were provided during

1967–90. Similarly, 599 *in-vitro* cultured materials and 16 984 seed samples were given mutagenic treatments during 1967–90. In recent years, the treatment of *in-vitro* materials has been preferred over that of dormant seed (see later), and treatment with fast neutrons has been preferred over that with gamma-rays (Brunner, 1992).

Seed/seedling Treatment

For the majority of studies on induced mutations in cereals and legumes, the seed is treated with an acute dose of physical or chemical mutagen, although a variety of pre-treatments and post-treatments may also be involved. In other cases, seeds may be germinated and seedlings are treated, even though such a treatment may lead to a higher rate of mortality. The treated seed/seedlings are used to raise M_1 generation. The seed is harvested on individual plant basis or individual spike basis and M_2 is raised in plant to progeny or spike to progeny rows. The segregation for induced mutations is examined in M_2 progeny rows.

There is, however, a basic difference in the origin of the germline from the cells in the embryos located in the seeds of cereals and legumes. The initials of tillers and, therefore, of the spikes (i.e. the germline) are already pre-determined as cells in the embryo of the cereals, but not in that of legumes (Gaul, 1965). In cereals, each tiller is derived from four or five cells in the embryo, so that if a mutation is induced in one or two of the four or five cells, chimaeric spikes may be obtained. In legumes, the flower primordia are not the direct descendants of meristematic cells in the embryo, but are the result of further differentiation and development. Consequently, the mutant cells in legumes may undergo diplontic selection and become eliminated before the origin of flower primoridia.

Treatment of Gametophytes

Mutagenic treatments have also been given to male and female gametophytes. For this purpose, in cereals, spikes are treated at the developmental stages ranging from uninucleate microspores to trinucleate pollen grains in case of the male gametophyte, and mature embryo sac in case of the female gametophyte. Unirradiated emasculated flowers are pollinated from irradiated spikes or vice-versa. Pollinated spikes and also unirradiated control spikes are bagged. Similar experiments can be designed for legumes also.

Experiments involving mutagenic treatments to gametophytes have been conducted in cereals such as barley, rice and wheat (Donini and Devreux, 1970; Devreux *et al.*, 1972). The major advantage of giving treatments to gametophytes is to overcome chimaerism, so that a plant derived from pollination after irradiation will be either heterozygous or homozygous, but cannot be a chimaera.

In both barley and wheat, it was shown that the female gametophyte has the higher level of radioresistance. However, it is recommended that if the spikes of cereals are treated at appropriate stages, and plants grown in isolation, this may not require emasculation and hand pollination. It was also shown that irradiation

of bi- or trinucleate pollen gives more mutations than that of the uninucleate microspores.

In-vitro *Techniques for Production, Selection and Fixation of Induced Mutations*

Techniques have also been developed for the production, selection (screening) and fixation of mutants in cell cultures. The most favoured induced mutations produced in cultured cells/tissues are those for adaptation to adverse environmental conditions. These adverse conditions include drought, heat, cold, freezing, soil salinity and low pH. Mutation breeding through *in-vitro* culture technology, therefore, can be used for complementing plant breeding. Plant and cell culture techniques are particularly suitable for mutagenesis, since they allow treatment and screening of large populations of cells in a short time and small space. It is possible to grow millions of cells in a Petri dish or in a flask, and irradiate (or treat with chemical mutagens) and multiply them on culture media to regenerate plants. In some cases the regenerating cells, somatic embryos and plantlets can also be subjected to heat, cold and freezing for selection of desired variants. The plants surviving these treatments can then be tested in the field. The use of *in-vitro* mutagenesis is, however, recommended mainly for vegetatively propagated crops and a CRP in this area has already been implemented by the Joint FAO/IAEA, Division.

In vitro culture techniques can also be used after giving a mutagenic treatment. For instance, in mungbean, the frequency of mutations could be increased when cotyledons were excised from irradiated seeds and raised *in vitro* to give M_1 generation. This was attributed to the additive effect of gamma-ray-induced variation and tissue culture-induced heritable variation (Mathews *et al.*, 1991). Plants from M_1 generation, raised after mutagenic treatment, can also be utilized for anther culture for fast homozygosity of induced mutations in the form of DH mutations (Maluszynski, 1990; Szarejko *et al.*, 1991). For this purpose, either seeds can be treated and used for raising M_1 generation, or spikes may be irradiated before they are used as the source of anthers for culture (Szarejko *et al.*, 1995). Dwarf, semidwarf, chlorina and uniculm mutations were successfully obtained in barley utilizing this approach (Umba *et al.*, 1991).

Direct Use of Induced Mutations in Major Genes/Quantitative Trait Loci (QTL) of Economic Value

Induced mutations for many economic traits have been used directly for improvement of many cereal and legume crops. Consequently spectacular success in mutation breeding work was achieved in self-pollinated annual cereals including crops such as wheat, rice, barley, oats, etc. However, similar success was not possible in grain legumes, as evident from the number of varieties produced in each crop (Table 1). One of the reasons for this is supposed to be relatively poor adaptation of the plant architecture in legumes to modern farming systems (Smartt and Hymowitz, 1985; Micke *et al.*, 1990). The plant architecture in grain legumes is

much more complex than in the cereals; therefore its inheritance should be complex. However, reconstruction of plant architecture in legumes, using single gene mutations, has been possible (FAO/IAEA, 1988), as has been demonstrated in chickpea (Shaikh *et al.*, 1980), pigeonpea and mungbean (Rao *et al.*, 1975), pea (Jaranowski and Micke, 1985), winged bean (Jugran *et al.*, 1986), and castor bean (Kulkarni, 1969).

During the past 25 years (1969–95), the major emphasis in cereals was laid on induced mutations for disease resistance and grain quality (protein). However, greater success was achieved for lodging resistance using short and/or stiff culm (FAO/IAEA, 1984c, 1988), crop duration or photoperiod sensitivity (Gottschalk and Wolff, 1983; Donini *et al.*, 1984; Konzak, 1984) and also grain protein (Micke, 1983; FAO/IAEA, 1979, 1984b; Müeller, 1984) (Table 2).

At Meerut University (India), we studied frequencies and spectrum of mutations induced by a variety of mutagenic treatments in: (i) cereals such as wheat, triticale, foxtail millet and barley, and (ii) legumes such as mungbean, urd, pea, lentils and chickpea. These studies led to the isolation of useful mutants for further genetic studies and for plant breeding. In mungbean, several high-yielding, disease-resistant mutants were subjected to yield trials leading to the release of a variety MUM-2 (Gupta *et al.*, 1994; Gupta, 1998).

Disease, Insect and Nematode Resistance in Cereals and Legumes

One of the most important mutants for disease resistance is M66 barley produced in 1942. This barley mutant is fully resistant to all pathotypes of powdery mildew and has not changed during the past 50 years. The mutant trait is monogenic and recessive (*ml-o* locus) and can be easily handled by breeders. Despite this the mutant could neither be used as a cultivar nor as a direct source of resistance, because alternative sources of disease resistance were available. This was due to its low yield potential (5–10% lower), and the presence of necrotic spots on leaf tips. Several other mutants for disease resistance had the same fate. The only mutant allele at *ml-o* locus used is *ml-09* induced by EMS, and incorporated in the cultivar '*Alexis*'.

It is now known that crop plants have defence genes which are distinct and different from the resistance and susceptibility genes. The resistance genes are known to initiate a signal transduction leading to the activation of defence genes (Ryan *et al.*, 1994; Staskawicz *et al.*, 1995). The *ml-o* is one such locus for resistance, which is known to regulate and foster the defence process. The attacked barley plant defends itself by sealing the infection sites through cell wall appositions made up of callose, an ability actually found in all barley varieties. The *ml-o* locus seems to act as a regulator, fostering this process, thus becoming effective against the fungus. This type of durable resistance should also occur in other cereals, and the mutants carrying such resistance should be included and used for breeding of cereals in general.

There are several other uses of induced mutations for disease and insect resistance leading to the development of new cultivars. 'CM 72' chickpea (Pakistan)

Table 2. Number of released varieties (cereals and legumes) developed through induced mutations and improved for individual traits

Species/crop	Induced mutations for characters*											Total
	1	2	3	4	5	6	7	8	9	10	Others	
Cereals												
Avena sativa (oat)	4	1	–	–	–	5	1	–	1	4	3	18
Hordeum vulgare (barley)	43	24	1	9	10	6	–	1	4	85	73	256
Oryza sativa (rice)	77	79	9	14	22	26	3	4	7	30	53	324
Secale cereale (rye)	2	–	–	1	–	–	–	–	1	–	–	4
Triticum aestivum	12	31	3	17	–	20	–	7	10	26	20	146
Triticum turgidum	10	–	–	1	–	–	–	–	–	06		
Zea mays	1	18	–	1	2	7	–	–	–	08	10	47
Legumes												
Arachis hypogea (groundnut)	4	10	–	1	–	1	–	–	–	18	04	38
Cajanus cajan (pigeonpea)	–	1	–	–	–	–	–	–	–	4	–	5
Cicer arietinum (chickpea)	–	1	–	–	–	3	–	–	–	4	–	8
Glycine max (soybean)	1	23	2	–	–	2	–	–	–	6	15	49
Phaseolus vulgaris (french bean)	–	5	–	–	–	3	–	–	–	3	10	21
Pisum sativum (pea)	6	5	–	5	–	–	–	–	–	2	14	32
Vicia faba (faba bean)	2	–	–	–	–	3	–	–	–	3	3	11
Vigna mungo (black gram)	1	–	–	–	–	–	–	–	–	2	–	3
Vigna radiata (green gram or mungbean)	–	8	–	–	–	3	–	–	–	1	–	12
Vigna unguiculata (cowpea)	1	–	–	–	1	–	–	–	–	7	–	9

*1, Plant height; 2, Duration; 3, Tillering/branching; 4, Lodging resistance; 5, Grain quality; 6, Disease/insect resistance; 7, Adaptability; 8, Drought/salinity resistance; 9, Cold/heat tolerance; 10, Yield and yield components.

(compiled from *Mutant Varieties Database*, updated to June 1996 and supplied by Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture)

and 'ICV 12' cowpea (Kenya) are two such examples. Induced mutations for disease resistance in wheat and variability for resistance against *Ascochyta* blight in chickpea were also reported recently (Sitch and Maluszynski, 1992).

Since, in some crops, there is evidence for the presence of susceptibility genes in addition to the resistance genes, disease resistance can also be achieved by either inactivating or deleting these genes for disease susceptibility. For instance, in wheat, it has been shown that there are genes on chromosomes 4B, 4D, 5B and 5D, which promote susceptibility in many wheat cultivars. Mutagenic treatments have been successfully used for inactivation/deletion of these genes. If this improved resistance is tagged using molecular markers (e.g. RFLP and RAPD), the same can be incorporated into a susceptible cultivar using molecular marker-aided plant breeding (Worland and Law, 1991). The successful use of RFLPs, while breeding for soybean nematode resistance, has been demonstrated recently (Young *et al.*, 1995).

Resistance to Abiotic Stresses (Drought, Frost, Salinity, etc.)

Induced mutants have been obtained in several cereal and legume crops for resistance to abiotic stresses such as drought, salinity, cold/frost, humidity, etc., leading to the release of many varieties (Table 3). However, induced mutants are uncommon for resistance to acid soil characterized by low availability of bases (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}), phosphorus deficiency and aluminium toxicity, although, some efforts in this direction have been made. For instance, in Brazil, aluminium tolerance in wheat was introduced using mutagenic treatments in variety Anahuac and in the F_4 generation of an interspecific hybrid between 6*x* and 4*x* wheats. These mutants should help in the development of varieties tolerant to aluminium (Al) toxicity for Brazil, where due to Al toxicity wheat production dropped from 6.1 million tonnes in 1987 to 2 million tonnes in 1993 (Camargo *et al.*, 1995). Molecular markers closely linked to Al tolerance are also being identified to help in molecular marker-aided breeding of Al-tolerant wheat and triticale cultivars (Somers *et al.*, 1995; Somers and Gustafson, 1996). Future efforts will be intensified in this direction, because acid soils cover approximately one billion (10^9) hectares of land in the tropical and subtropical regions of the world.

Dwarf Mutants in Cereals

Semi-dwarf mutants in cereals contributed greatly to the green revolution in the 1960s, and because they could withstand and respond to high fertilizer and water application, were resistant to lodging (Athwal, 1971). Although, the genes for semi-dwarfing occur in nature and could be utilized in breeding (e.g. 'Norin' and 'Seu Seun' in wheat and 'dee-geo-woo-gen' or 'DGWG' in rice), a large number of dwarf mutants in a variety of cereals were also produced through induced mutations and utilized in breeding programmes. For instance, lodging-resistant semi-dwarf and high-yielding varieties including 'Reimei' (derived from 'Fujiminori')

Table 3. Mutant varieties showing resistance to different abiotic stresses

	Drought	Salt	Cold/frost	Humidity
Arachis hypogea	Changhua No. 4	–	Changhua No. 4	–
Cajanus cajan	Co5	–	–	–
Cicer arietinum	–	Kiran	–	–
Gycine max	Heinong No. 6, Heinoun No. 16 Cerag No. 1		Cerag No. 1	Heinoun No. 8
Hordeum vulgare	Balder J., Safir RD-137, Anni	–	Jutta, Radikal, Prisiv, Novator, Pennrad, Akkord, Boyer, Diana, Yubilei 100, Grammos, Vovilon	–
Lathyrus sativus	Poltavskaya 2		–	–
Oryza sativa	202, RD15, CNM6 Danau atas	Shadab, 6B Fuxuan No1, Atomita 1 Atomita 2 Liaoyan 2 Mohan = CSR4 A-20, Shua 92	Wanhongfu, Zaoyeqing, Qikesui, M112, M114, Xindao no. 1, Xiangfudao, Wanfu 33, Fuxiang No. 1, Shuangke No. 1 Reimei, Nongshi No. 4, Fu 709, Ibukiwase, Guifu No. 3, Hongnan, Fuzhu, Houhai, Xiushui 48	
Secale cereale	–	–	Hankkija's Jussi	–

(based on *Mutant Varieties Database*, FAO/IAEA, 1996)

Table 3. Mutant varieties showing resistance to different abiotic stresses (contd)

	Drought	Salt	Cold/frost	Humidity
Triticum aestivum	Zhengliufu Yuyuan No. 1 Yunfu No. 2 Longfumai No. 3 Wuchun No. 3 Taifu Nos. 1, 10, 15, 22, 23, Qichun No. 1, Jienmai No. 2, Xifu 4, Zlatostrui, Xinchun No. 2, Omskaya ozimaya	Emai No. 9 Changwei 19 Changwei 51503	Albidum 12, 503, 1161, Changwei 51503 Jiaxuan No. 1, Zhonga No. 1, Eritrospermum 103, Yuanfeng Nos. 1, 2, 3, Meshenskaya, Silbirskaya niva, Yuandong No. 772, Kharkovskaya 90, Polukarlik 3	79p-17
Vigna radiata	Co4	Camar	–	–
Zea mays	Luyuan SC No. 4	–	Longfuyu No. 1 (hybrid)	–

(based on *Mutant Varieties Database*, FAO/IAEA, 1996)

and 'Basmati' (derived from 'HR-47') in rice; 'Pallas' (derived from 'Bonus') and 'Golden Promise' (derived from 'Maythorpe') in barley resulted from the use of induced mutations (Futsuhara *et al.*, 1967; Reddy and Reddy, 1971; Borg *et al.*, 1958).

A number of semi-dwarf oat mutants were isolated by Konzak (1993) in M_2 and M_3 generations following treatment of seeds with EMS + sodium azide. Selected mutants are being analysed for their potential as cultivars for commercial cultivation. Mutations for short culm were also obtained in rice, which were subjected to yield trials in Bangladesh (Shwe and Shaikh, 1993).

Earliness for Flowering/Maturity

Earliness for flowering/maturity is another important trait which is desirable in several crops. A large number of mutations for earliness have been obtained leading to release of early varieties in several cereal and legume crops (e.g. groundnut, oats, chickpea, soybean, barley, rice, pea, wheat; see FAO/IAEA's *Mutant Varieties Database*, 1996).

Photoperiod Insensitivity

Photoperiod sensitivity of several varieties of cereals does not allow their adoption in other regions of the world due to variation in photoperiod during the growing season. Induced mutations, for day length insensitivity have been induced leading to release of day length-insensitive cultivars in rice (Calmochi-101, M-202, M-203, M-204, Biraj, Savitri, Intan Mutant, RD-10, Xiushui 48, Xianghu 24) and pigeonpea (Co5).

Mutants for Protein Content, Grain Quality

Several studies are available on induced mutations for increasing seed protein content, although increase in protein content often led to reduced grain yield. Such studies have been conducted in wheat and rice and also in some pulse crops including mungbean (Bhagwat *et al.*, 1979; Narahari and Bhatia, 1973; Narahari *et al.*, 1976; Bhatia and Mitra, 1982). The mutant cultivars with increased protein content are listed in the FAO/IAEA *Database* and include the following: oats (Nasta), soybean (Bisser, Boriana, Heinong 28), barley (Prisiv, Moskovskii 2, Fakel, Shua), rice (Iratom 38), pea (Moskovsky 73, Nemchinovskii 85), wheat (Humai No. 3, Carolina, Yuanfeng No. 5, Soghat 90), maize (KNEJA 510, KNEJA 666, KNEJA-HP-556, Longfuyu No. 1).

Mutations for improved grain quality have also been obtained in barley, wheat, maize, rice, oats, soybean, etc. These also include macro-mutants such as opaque-2 and floury-2 having higher lysine content. In wheat, variability for lysine content and bread-making quality is low and an approach for inducing micromutations has been tried leading to the release of variation for different protein fractions (Bhatia *et al.*, 1970; Pogna *et al.*, 1995). More recently a transgenic

approach has been used in wheat to improve protein quality (Blechl and Anderson, 1996; Altpeter *et al.*, 1996).

In barley, brewing quality and malting quality have been improved through induced mutations leading to the release of several varieties (see FAO/IAEA *Mutant Varieties Database*, 1996). Several value-added barley strains for the brewing industry have been obtained through induced mutations. For instance, several mutants that were free of red anthocyanin pigment and proanthocyanidin (*ant 13*, *ant 17*, *ant 18*, *ant 21*, *ant 22*) were isolated (presence of pigment requires stabilization of beer against haze formation). In a recent study, 560 such mutants were selected by screening 18.5 million M_2 plants. Several of these mutants were also high-yielding. The testing of these mutants led to the release of two varieties, namely '*Caminant*' and '*NFC 8808*'. Another proanthocyanidin free mutant, *ant 29-2110*, outyielded the standard mixture of *Alexis* and *Canut* (Von Wettstein, 1995).

Use of barley in malting technology also involves a kilning process, which is the last stage in malting and involves drying of the germinated grains at 80°C. At this temperature, the (1–3), (1–4)-β-glucanase enzyme, necessary for degradation of (1–3), (1–4)-β-glucans (the main constituents of endosperm cell wall), does not survive. This leads to an unacceptable level of viscosity for filtration or centrifugation of wort. Heat-stable (1–3), (1–4)-β-glucanase will therefore give value-added barley not only for the beer industry but also for its use as a feed for chicken and young piglets, who cannot digest β-glucans. Transgenic barley plants with a gene for heat-stable β-glucanase are being produced for this purpose (Von Wettstein, 1995).

Induced mutagenesis has also been used for eliminating unwanted fatty acids and/or for elevating preferred fatty acids. For instance, high palmitate or high stearate soybean varieties were derived using induced mutations (Bubeck *et al.*, 1989). A series of low stachyose lines suitable for animal feed have also been developed through induced mutations (Kinney, 1995). Transgenic approaches have also been used recently for alterations of fatty acid composition of edible seed oils (Knutzon *et al.*, 1992; Gibson *et al.*, 1994; Kinney, 1995).

Indirect Use of Mutagenic Treatments

Improvement of Hybrid Rice Through Induced Mutations

In China, hybrid rice is being grown in large areas (8 million hectares in 1986, 17.3 million hectares in 1991; Yuan and Fu, 1995). Hybrid rice gave yield advantage of 20–30% (an increase of 1.0–1.5 t/ha). However, these hybrids need longer growth duration (125–140 days), lack disease/insect resistance and exhibit poor grain quality unacceptable to the world market. Induced mutations were successfully utilized to overcome some of these problems (Mingwei, 1989) by producing restorer lines such as 'Fuhui 06' that was 20 days earlier and 10 cm shorter (Xiao, 1983) and 'Fu 36–2' having better grain quality,

adaptability and resistance to blast. Similarly, induced mutations were used to obtain improved male sterile lines such as 'Hontou 31A' having good grain quality and desirable flowering habit (Abao, 1986). In Japan a thermosensitive genetic male sterile rice variety 'Norin PL12' was also developed for hybrid rice (FAO/IAEA, 1996).

Induced mutations involving flower structure more suitable to cross-pollination were also obtained and used for hybrid seed production in rice (Li *et al.*, 1981; Yang and Haoran, 1986; Shaokai *et al.*, 1989) and barley (Ulrich *et al.*, 1989).

'Mutant Heterosis' in Cereals

It has been shown that heterosis may appear in crosses between two mutants derived from the same parent variety or in crosses of a mutant with the parent variety. A statistically significant effect of heterosis in these crosses was described for characters such as yield (seed production, green matter production), plant height, leaf and flower size, tillering, root system, seed protein content and net assimilation rate. Often the heterotic effect ranged from 30% to 100% above the best parent. It was also shown that even mutants with extremely poor agronomic characters can give excellent F_1 plants, outyielding a parent variety. This phenomenon has been described as 'mutant heterosis', but its exploitation has been limited due to lack of genetic systems for hybrid seed production in several crops. In crops such as barley and wheat, the F_1 hybrid did not always outyield the best available cultivars, thus questioning the utility of hybrid wheat production. However, when one or both the parents of a cross were mutants, the yield of F_1 significantly exceeded that of the best parent (Ramirez, 1969; Konzak, 1989; Maluszynski *et al.*, 1988a,b, 1989).

In maize, it has been shown that an additive genetic system is involved in the mutant heterosis, so that it should be theoretically possible to obtain homozygotes equivalent to F_1 hybrids in their performance. For this purpose a doubled haploid system can be used for fixing the heterosis. Maluszynski and Szarejko (1994) proposed a scheme for the production of 'F_1 performing' doubled haploid lines (Fig. 1). Following this scheme in barley, 'F_1 performing' doubled haploid lines have been successfully obtained from heterotic hybrids (F_1). DH_3 lines, which yielded only slightly lower than F_1 hybrids, but significantly exceeding the parent variety, were actually obtained.

Disrupting Negative Association Between Two Desirable Traits in Soybean

There are also examples where mutagenic treatments were used to break negative association between two desirable traits. For instance, in soybean, Bhatnagar *et al.* (1992) reported disruption of negative association between oil content and protein content. This negative association between high oil and high protein content is well documented, and any effort to disrupt this negative association or to establish a positive association should be welcome.

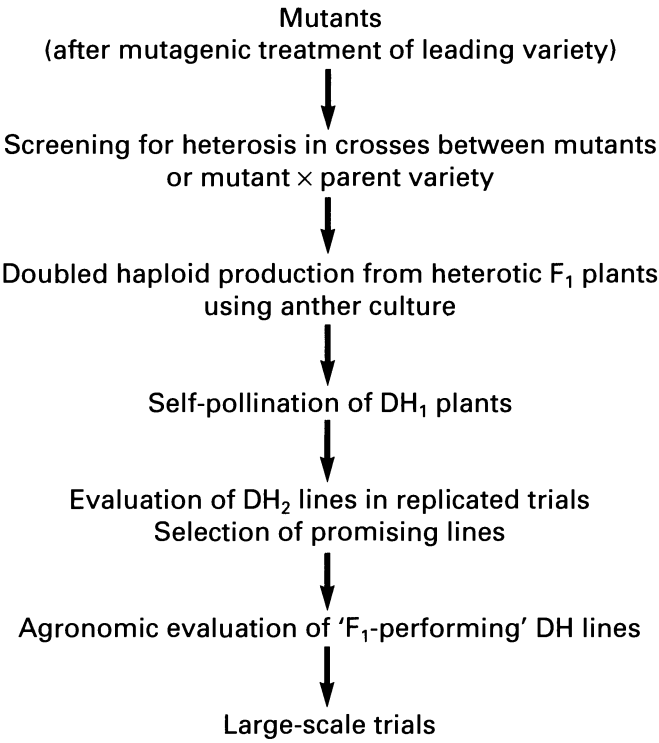

Figure 1. Scheme for production of F_1-performing doubled haploid lines from heterotic hybrids derived from crosses of induced mutants (from Maluszynski and Szarejko, 1994)

DNA-based Marker Mutations for Improvement of Cereals and Legumes

The speed and efficiency of plant breeding can be improved with the help of DNA-based molecular markers, that are closely linked to genes of interest. The expression of these genes of interest may depend on environment, thus preventing effective selection, or may be delayed in development, thus preventing selection at the seedling stage. Keeping this in view, FAO/IAEA launched a CRP entitled 'Use of DNA-Based Marker Mutations for Improvement of Cereals and Other Sexually Reproduced Crop Species', in 1991. At the Research Coordination Meeting (RCM) in 1992, the application of molecular marker techniques for the following purposes was demonstrated (Sitch, 1992): (i) germplasm characterization and parental selection; (ii) varietal protection; (iii) improved efficiency of backcross breeding involving introgression of novel genes from exotic germplasm, and (iv) tagging of important genes for marker-aided selection. At the above RCM, successful tagging of several genes was also reported (Table 4).

Morphological mutants can be included in DNA marker-based genetic maps. Therefore, the generation of mutagenesis-derived resources for molecular analysis and genetic mapping activities in wheat and *Vigna* will receive major attention of

Table 4. Genes tagged and targeted to be tagged with molecular markers in some crop plants

Crop	Genes already tagged	Genes targeted to be tagged
Vigna radiata	Resistance to bruchids, powdery mildew, a major gene for seed weight (QTL)	Virus resistance, seed weight
Vigna unguiculata	Cowpea chlorotic mottle virus, a major gene for seed weight (QTL)	–
Oryza sativa	Dwarfing gene (*sd1*)	Aroma, grain quality, earliness, semi-dwarfism, thermosensitive, male sterility
Zea mays	–	Aluminium tolerance
Sorghum	–	Resistance to downy mildew and aphid, plant height, panicle morphology

(from report FAO/IAEA's RCM, 1992; Sitch, 1992)

the above CRP of FAO/IAEA. Deletion mutants are particularly useful for gene cloning using strategies such as genomic subtraction. A large number of such deletions have become available in wheat (Endo and Gill, 1996). DNA markers can also be used for facilitating selection of desirable translocations induced by irradiation for transfer of desirable genes from wild species into a crop such as wheat (Jiang *et al.*, 1994). Several other traits that can be used for tagging include the following: aroma, grain quality, earliness, semi-dwarfism, thermosensitivity, male sterility (in rice), aluminium tolerance (in maize), virus resistance and seed weight (in *Vigna*).

Induced Structural Changes

While creation of new alleles due to base substitutions in DNA is often the most common source of induced mutations, chromosome interchanges are also induced by mutagenic treatment. A large number of such interchanges covering a variety of breakpoints were produced in barley at Svalöf, Sweden, by A. Hagberg and his colleagues. These translocations, when crossed with each other, give duplications in a small frequency, so that a good collection of duplications is now available at Svalöf. Some of these duplications in barley were found to be high yielding and can be used in plant breeding (Hagberg and Hagberg, 1991).

Transfer of Desirable Traits from Wild Species

Mutagenic treatments have also been extensively used for the transfer of desirable traits from wild species to crop plants including several cereals such as wheat (Friebe *et al.*, 1996). Several useful gene transfers have been achieved through either radiation-induced translocations or through recombination between wheat chromosomes and the alien chromosomes (Table 5). Similar transfers have been successfully tried in other cereals also (rice, oats, etc.).

Future Prospects

The conventional method of induced mutations played a significant role in the production of improved cultivars of both cereals and legumes. Several recommendations for future mutation breeding were, however, made recently at IAEA meetings (Anonymous, 1996a,b). These recommendations include the following: (i) the use of doubled haploid (DH) lines for speeding up the variety production programme using induced mutations; (ii) development of rapid screening and evaluation techniques for stress tolerance; (iii) the study of genetics of stress resistance; (iv) development of molecular markers closely linked to stress resistance and their use in pyramiding genes; (iv) increased use of mutant heterosis; and (v) development of a mutation database on Internet for easy and quick access.

The conventional approach of mutation breeding will continue to be used in the future, keeping above recommendations in mind. However, this will be supplemented by a variety of new approaches available for altering the genetic constitution of crop plants by introducing genetic variability missing in the primary gene

Table 5. Alien genera/species and the alien genes transferred through irradiation/recombination for improvement of bread wheat

Alien species (donor)	Genes transferred
Aegilops	
Ae. umbellulata	*Lr9*
Ae. speltoides	*Lr28, Sr32, Lr35/Sr39, GB5*
Ae. longissima	*Pm13*
Ae. comosa	*Yr8/Sr34*
Agropyron	
A. elongatum	*Lr19/Sr25, Lr19, Lr29, Sr24/Lr24, Sr26, WSMR, Cmc2*
A. intermedium	*Wsm1, Lr38, Sr, BYDR*
Secale	
S. cereale	*Pm8/Sr31, Lr26/Y49, Gb2/Pm17, Gb6, Lr25/Pm7, Lr45, Sr27, Pm20, H21, H25*
Triticum	
T. timopheevii	*Sr36/Pm6, Sr37, Lr18, Sr40*

Source: (Friebe *et al.*, 1996)

pools. This will be done by production of transgenic crops and by other novel methods of mutagenesis. For instance, a number of mutant genes (produced by T-DNA insertion mutagenesis) have been isolated from genomes of a variety of plant species (Feldman, 1991; Koncz *et al.*, 1992). These can be introduced in their original form or in modified form for improvement of a variety of economic traits in cereals and legumes. An important approach is to utilize normal or mutant genes from *Arabidopsis* (which has been extensively used for mapping and cloning of genes) for the production of transgenic crops. For instance, two important mutant genes (isolated from *Arabidopsis*) for altered epicuticular wax (EW), important for imparting resistance to herbivorous insects, have been introduced in *Brassica oleracea*. Other mutant *Arabidopsis* genes that are becoming available for improvement of crop plants include the following: (i) constructs of mutant genes, *fad2* and *fad3* (encoding fatty acid desaturases) for manipulating soybean oil; (ii) gene for uptake of nitrate, *CHL1* (already cloned) for manipulating nitrate uptake; (iii) genes involved in ethylene production for breeding plants resistant to environmental stresses (for a recent review see Feldman, 1995).

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17. Induced Mutations in Ornamental Plants

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Introduction

Ornamental plants are ideal for the application of mutations induction techniques because many economically important traits, e.g. flower characteristics or growth habit, are easily monitored after the mutagenic treatment. Furthermore, many ornamental species are heterozygous and often propagated vegetatively; this allows the detection, selection and conservation of mutants in the M_1 generation. Not surprisingly, for centuries breeders have made use of spontaneously occurring sports, which have contributed extensively to the generation of diversity in ornamental species. For example, the origin of the moss rose was first observed in 1696 as a mutant of *Rosa centifolia* (Hurst and Breeze, 1922); among 5819 rose cultivars marketed during 1937–76, 865 were developed from bud mutations (Haenchen and Gelfert, 1978). In azaleas and chrysanthemums, approximately 50% of cultivars have been derived from natural sports or induced mutations (Heursel, 1980; Preil, 1986). Since the 1930s mutation induction has been applied to ornamental plants. The first commercial officially released mutant was in tulip, cultivar Faraday, with altered flower colour resulting from irradiation of cv. Fantasy by De Mol in 1936 (Broertjes and van Harten, 1988). In recent decades, the development of 'sport-families' by mutagenesis has become routine practice for several important ornamental genera, such as *Dendranthema*, *Dianthus* and *Euphorbia.* Breeders were particularly interested in producing flower colour mutants from outstanding cross-bred products, even before the original genotype was distributed for marketing (Broertjes *et al.*, 1980), because former plant breeders' rights regulations allowed the discoverer of a sport to exploit it commercially. Now the situation is changing in an increasing number of countries following the UPOV convention of 1991 that allocated the variety rights of every sport to the breeder of the original cultivar.

Ornamental plants comprise diverse groups including flowering and foliage pot plants, cut flower crops, tuber and bulb crops, annual and perennial garden plants as well as trees and shrubs. Mutation induction techniques have been applied to all these groups. Broertjes and van Harten (1988) comprehensively reviewed and listed about 100 species which have been subjected to experimental mutation induction. According to the available information, about 500 mutant cultivars in 29 ornamental species have been officially released (Broertjes and van Harten, 1988; Maluszynski *et al.*, 1992; Anonymous, 1994, 1996; Bhatia, 1991; Kawai and Amano, 1991; Wang, 1991). However, the real number of such varieties is much greater than the reported number because: (1) most commercial breeders are unwilling to disclose the methodology in developing new cultivars, and (2) mutation

S.M. Jain, D.S. Brar, B.S. Ahloowalia (eds.), Somaclonal Variation and Induced Mutations in Crop Improvement, pp. 333–366.

induction is now a routine technique and no longer the subject of many scientific publications.

Induced mutations in ornamentals comprise traits, such as altered flower characters (colour, size, morphology, fragrance); leaf characters (form, size, pigmentation); growth habit (compact, climbing, branching); and physiological traits such as changes in photoperiodic response, early flowering, free-flowering, flower keeping quality, and tolerance to biotic and abiotic stresses. This chapter deals with 'classical' mutagenesis, i.e. alteration of a specific DNA constitution by treatment with chemicals or ionizing radiation. T-DNA or transposon insertion mutagenesis is still restricted to a few model systems. The potentials and drawbacks of these techniques in improvement of ornamentals have recently been discussed by Jenks and Feldmann (1997). The achievements and unsolved problems associated with genetic engineering are beyond the scope of this chapter. They will be briefly discussed with regard to the future impact of 'classical' mutagenesis on improvement of ornamental plants.

Mutations in Flower Colour

There are numerous reports on alterations of flower characteristics of ornamental species arising after mutagenic treatment. The literature cited by Broertjes and van Harten (1988) and the publications during the past 10 years show that 55% of the records concerned changes in flower colour and 15% in flower morphology. Table 1 lists ornamental species for which induced mutations of flower colour have been reported. The majority of relevant publications appeared prior to the mid-1980s, and have been discussed by Broertjes and van Harten (1988). We avoid repetition and refer to their review as B&vH in the text of this chapter.

Three major groups of pigments are known to be responsible for flower colour: (1) flavonoids, (2) carotenoids, and (3) betalains. Factors such as vacuolar pH, copigmentation, formation of metal complexes and the shape of epidermal cells, contribute to the final expression of flower colour. Considerable progress has been made in elucidating the biosynthesis and genetics of pigments (Forkmann, 1993; Forkmann *et al.*, 1995; Davies and Schwinn, 1997). Especially for the flavonoids, several genes encoding important biosynthetic enzymes have been isolated and used for genetic transformation of some ornamental species. Progress on the molecular aspects of biosynthesis of carotenoids and betalains has been made as well (Davies and Schwinn, 1997). In spite of the available molecular knowledge, most of the efforts in altering the flower colour by mutagenesis have been undertaken from an empirical point of view. The majority of reports in Table 1 are purely descriptive and devoid of analytical data on mutated genes.

Sport Families in Herbaceous Species

In several ornamental crops, successful attempts have been made to induce a wide range of flower colour, starting from a selected genotype. Such sport-families

Table 1. Induced mutations in flower colour*

Genus	Reference
Alpinia	Soedjono, 1992
Alstroemeria	B&vH†; MBNL,+ 1992
Amaryllis	B&vH
Begonia	B&vH; Benetka, 1987; Soedjono, 1988
Brachycome	Walther and Sauer, 1989b
Calathea	B&vH
Callistephus	Wosinska, 1986
Canna	B&vH; Khalaburdin, 1991; MBNL, 1992
Dahlia	B&vH
Dendranthema	B&vH; Nikaido and Onozawa, 1989; Matsumoto and Onozawa, 1989; Jerzy, 1990; Antonyuk, 1991; Datta, 1991a; Nagotomi, 1991; MBNL, 1992; Ahloowallia, 1992; Nagatomi *et al.*, 1993; Jerzy and Zalewska, 1996; Tulmann Neto and Latado, 1996
Dianthus	B&vH; Silvy and Mitteau, 1986; MBNL, 1992; Simard *et al.*, 1992; Cassells *et al.*, 1993
Euphorbia (fulgens)	B&vH
Eustoma	Nagatomi *et al.*, 1996
Forsythia	B&vH; van de Werken, 1988
Geranium	MBNL, 1992
Gerbera	Walther and Sauer, 1986a, 1990; Laneri *et al.*, 1990; Jerzy and Zalewska, 1996
Gladiolus	B&vH; Raghava *et al.*, 1988; Sedelnikova, 1988; MBNL, 1992; Zhakote and Murin, 1994
Hibiscus	B&vH; MBNL, 1992
Hyacinthus	B&vH; MBNL, 1992
Iris	B&vH
Kalanchoe	B&vH; Schwaiger, 1992
Kohleria	B&vH; Geier, 1988
Lantana	Datta, 1991a, 1995
Lilium	B&vH; Grassotti *et al.*, 1987
Muscari	B&vH
Paeonia	Uspenskaya, 1993
Pelargonium	B&vH
Petunia	Padjama and Sudhakar, 1987; pers. comm. by German breeders
Phlox	Antonyuk, 1991
Portulaca	B&vH
Rhipsalidopsis	B&vH
Rhododendron	B&vH; MBNL,1992
Rosa	B&vH; Zykov and Klimenko, 1988; Antonyuk, 1991; Datta, 1991a; MBNL, 1992; Kaicker, 1992; MBNL, 1996
Rudbeckia	Shukla *et al.*, 1986
Saintpaulia	B&vH
Sinningia	Paek and Han, 1988
Streptocarpus	B&vH; MBNL, 1992
Tulipa	B&vH
Weigela	B&vH; Duron, 1992

Table 1. Induced mutations in flower colour* (contd)

Genus	Reference
Zinnia	Venkatachalam and Jayabalan 1991, 1994a
Zygocactus	B&vH

*The type of colour alterations could not be included. The magnitude of details would be beyond the scope of the table.

†B&vH = Reports mentioned by Broertjes and van Harten (1988).

‡MBNL = *Mutation Breeding Newsletter*, list of cultivars.

offer the horticultural advantage of growing them under identical environmental conditions. The most prominent example is chrysanthemum (*Dendranthema* × *grandiflorum*), in which hundreds of new cultivars have been developed by mutation induction throughout the world. Jank (1957) evaluated 2300 cultivars marketed during the period 1843–1954, and found that the pink genotypes regularly gave rise to numerous sports, that included most of the colours seen in chrysanthemum. Conversely, cultivars with golden-orange flowers seldom sported, exclusively resulting in yellow petals or red ones with yellow undersides. These findings have been reconfirmed many times and are subsumed in Figure 1.

For breeders intending to create flower colour variability by mutation induction in species other than chrysanthemum, the knowledge of putative direction for

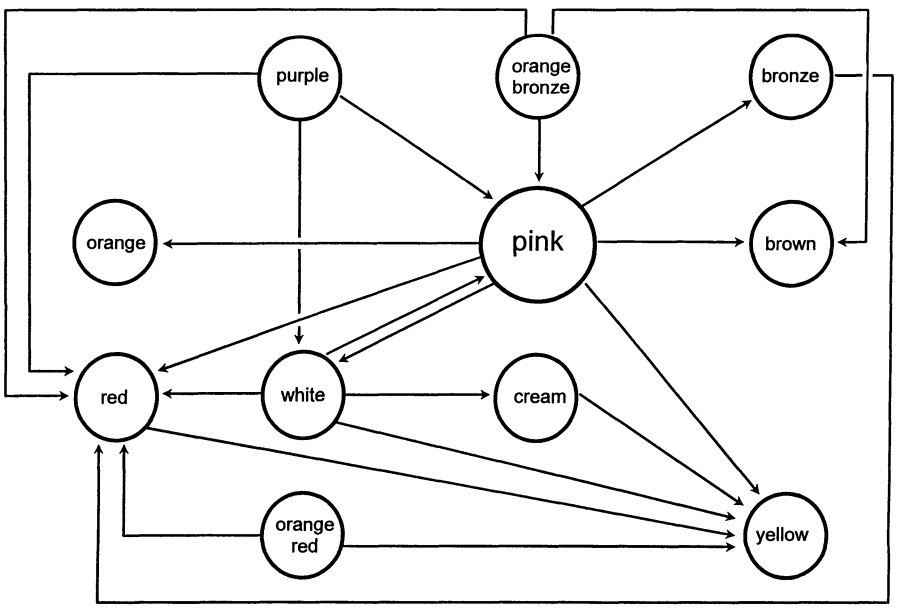

Figure 1. Main derivations of flower colour mutations in chrysanthemum (data from Jank, 1957; Broertjes, 1966; Jordan and Reimann-Philipp, 1983)

mutations would be helpful in selecting the appropriate 'parent' genotypes. In most cases a shift from dominant to recessive alleles is expected; however, the situation is more complex. In addition to single-point mutations of genes that are involved in the pigment biosynthetic pathway, most probably more than one gene will be affected. Furthermore, changes in the regulatory genes, chromosomal aberrations, and chimaerism leading to specific colours or rearrangement of preexisting genetically diverse layers can contribute to phenotypic variation.

Another example for an intensive exploitation of sport-families, obtained either by spontaneous or by induced mutations, is seen in *Dianthus*. Similar to chrysanthemums, many of the investigated mutant genotypes were periclinal chimaeras (Broertjes and van Harten, 1988). Silvy and Mitteau (1986) listed 19 flower colour mutants, derived from cv. Pallas, and submitted for registration in France during 1981–84. After mutagenic treatment of the original cv. Pallas (homohistont: LI, LII, LIII 'yellow with red streaks') and its mutant 'Pallas Orange' (periclinal chimaera: LI 'orange with red streaks', LII and LIII unchanged), Silvy and Mitteau (1986) found flower colour mutants with a frequency of 0.8% and 3.3%, respectively. They assumed a mutation direction from yellow with red streaks to pure yellow, followed by red and pink. Furthermore, Silvy and Mitteau (1986) compared different methods of mutation induction, i.e. the application of acute doses with or without low predoses and chronic irradiation of *in-vitro* and *in-vivo* material. They concluded that for carnations the most promising method was acute irradiation of *in-vivo* rooted cuttings followed by pruning of the plants. Working with petal explants *in vitro*, Simard *et al.* (1992) produced most mutants by X-ray treatment on the fourth day of culture, which they found to be the time of dedifferentiation of cells giving rise to adventitious buds. Starting with the red-purple genotype 'Mystère', Cassells *et al.* (1993) selected about 2% useful flower colour variants ranging from light pink to dark purple-red in plants regenerated from *in-vitro* nodes, treated with different doses of X-rays followed by three successive subcultures.

Walther and Sauer (1986a, 1991) suggested the development of sport-families in *Gerbera* by *in-vitro* mutagenesis and used, among others, a heterozygous genotype with lilac-red inflorescences carrying a dark-coloured centre. This latter trait is inherited in a monogenic dominant manner and is, therefore, an ideal marker gene in mutation induction studies. After X-ray treatment of *in-vitro* shoots, in addition to mutants of this marker, a considerable variation in flower colour was obtained ranging from red to different shades of violet-pink up to cream. Laneri *et al.* (1990) found six different flower colour mutants and 13 types of variations affecting flower morphology following irradiation of *in-vitro* shoots of a pink cultivar. Although, there have been considerable efforts to optimize methods for *in-vitro* mutagenesis in *Gerbera*, e.g. by analysis of radiosensitivity of genotypes or by using fractionated doses (Dubec-Lebreux and Vieth, 1987a,b; Walther and Sauer, 1989a, 1990, 1992) as well as by the use of *in-vitro* adventitious bud techniques (Jerzy and Lubomski, 1992), only one cultivar derived from mutagenic treatment has been officially registered, (Jerzy and Zalewska, 1996). Sport-families with induced changes in flower colour have been produced in other

species as well, including *Dahlia*, *Alstroemeria* and *Streptocarpus*. As far as we know, in addition to literature discussed by Broertjes and van Harten (1988), no recent publications are available.

Woody Ornamental Plants

Mutation induction is especially advantageous for breeding woody species due to long vegetative cycles. In *Forsythia*, induced variation of yellow colour has been described (B&vH), and at least one new cultivar with altered flower colour, 'Lemon Screen' bearing lemon-yellow flowers, has been released (Van de Werken, 1988). By mutagenesis in *Hibiscus*, a new cultivar 'Shirasagi-no-Yume' has been marketed in Japan (Maluszynski *et al.*, 1992). In *Rhododendron simsii*, 15 cultivars with new flower colours, derived from mutation induction, were released mainly during the 1970s (Heursel, 1980; Maluszynski *et al.*, 1992). Most of these alterations also appeared as natural sports. De Loose (1979) outlined the direction of sporting of flower colour in azaleas (Fig. 2). No further work has been published. In *Lantana depressa*, two flower colour mutants, i.e. canary yellow 'Niharika' and 'L. depressa bicoloured', with yellow and white flowers have been marketed (Datta, 1991a, 1995). A new *Weigela* cultivar 'Courtavif Rubivif', with a brighter red flower colour has been selected in France after mutagenic treatment of rooted cuttings (B&vH). Later, the same group treated *in-vitro* shoots (Duron and Decourtye, 1986) and *in-vitro* stem segments (Chevreau *et al.*, 1990; Duron, 1992) with gamma-rays or ethylmethanesulphonate, respectively, and found among other mutated traits variation in flower colour in chimaeral as well as in non-chimaeral plants.

Intensive mutation induction work has been done with *Rosa*, and at least 35 new cultivars with altered flower colour have been marketed (B&vH, Maluszynski *et al.*, 1992; Anonymous, 1996) after treatment of either *in-vivo* or

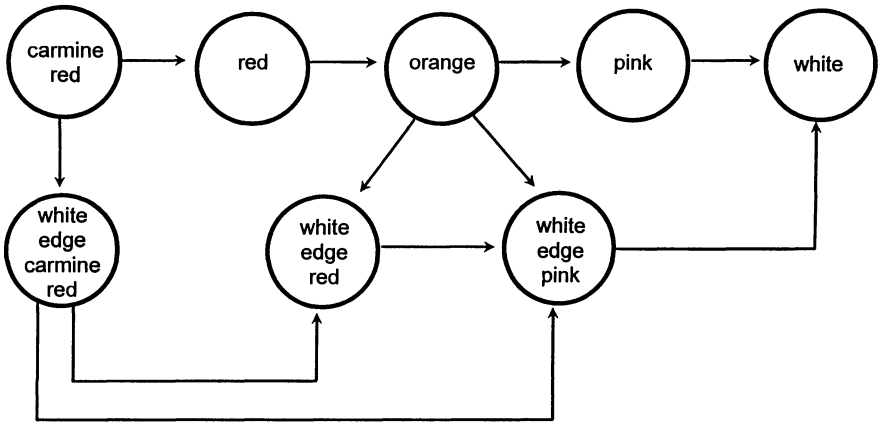

Figure 2. Main derivations of flower colour sporting in *Rhododendron simsii* (modified after De Loose, 1979)

in-vitro material. For example, great variability was induced by applying X-ray doses between 25 and 60 Gy to nodal segments of *in-vitro*-grown shoots (Walther and Sauer, 1986b). Of the mutants produced by applying higher doses, 73% were related to flower characteristics. Mutation breeding work with roses in India has been summarized by Datta (1991a) and Kaicker (1992). Several reports from the Ukraine deal with rose breeding strategies, including mutagenesis (e.g. Zykov and Klimenko, 1988, 1989, 1993a). Recent reports comprised attempts to characterize rose mutants by analysis of flower colour pigments (Datta, 1991b; Zykov *et al.*, 1990, 1991, Zykov and Klimenko 1993b).

Tuber and Bulbs

No new mutation breeding activities in tuber and bulb ornamentals have been published in addition to the literature discussed by Broertjes and van Harten (1988), except for *Gladiolus* and *Lilium* (Table 1). Gamma-radiation treatment of dormant corms of the white flowering *Gladiolus* cv. White Friendship gave several plants which showed sectorial pink flower coloration. A mutant with lighter pink florets was isolated in pure form from the M_1V_3 generation (Banerji *et al.*, 1994).

Seed-propagated Species

The application of mutation induction techniques has been limited in seed-propagated ornamentals as compared to vegetatively propagated species. In *Zinnia*, seed treatment with gamma-rays led to a considerable degree of variation in flower colour in the M_2 generation. The crimson red of the original genotype changed to magenta, yellow, red, and red with white spots (Venkatachalam and Jayabalan, 1991). Mutant traits were passed on to the next seed generations. The magenta mutant segregated in 3:1 ratio (magenta and yellow) in the M_3 generation (Venkatachalam and Jayabalan, 1994a). In *Eustoma*, explants from petals and leaf blades were cultured *in vitro* after chronic irradiation of young plants of two cultivars. Repeated selfing of regenerated mutants gave three genotypes with new attractive ornamental traits, including alteration of flower colour (coloured marginal variegation), which were submitted for registration (Nagatomi *et al.*, 1996).

Flower Colour Chimaeras

Chimaeras can arise by mutagenic treatment, and this depends on the type of material used and the regeneration process. In contrast to other traits, flower colour mutants can readily be recognized even if very small sectors are affected. For example, the size of mutant sectors varied from narrow streaks on single petals to whole flowers after the irradiation of rose budwood (Datta, 1989). Due to their location and limited size, in many cases such mutations are difficult to isolate by traditional methods, such as repeated cutting back of plants. Therefore, attempts were made to isolate solid mutants by *in-vitro* culture of explants carrying the

mutated sector. In chrysanthemum, small sectored mutations were successfully secured by regeneration of adventitious shoots from sported regions of petal tissue (Nikaido and Onozawa, 1989; Malaure *et al.*, 1991b, Nagatomi *et al.*, 1993). Care has to be taken with regard to restricting the amount of possible concomitant somaclonal variation due to callusing of the explants (Malaure *et al.*, 1991a,b). Segregation of chimaeric tissues has also been achieved by initiation of cell suspension cultures and subsequent plant regeneration in *Euphorbia pulcherrima* (Preil and Engelhardt, 1982). In *Gerbera*, small sectored flower colour mutants were separated by *in-vitro* plant regeneration from corresponding capitulum explants (Schum, unpublished). The histological analysis of carnation petals, grown *in vitro*, showed that buds were formed from both epidermal and subepidermal cells. Thus, regeneration of adventitious shoots can reveal the genotype of the outer as well as the internal cell layer (Simard *et al.*, 1992). This applies also for other plant species for which appropriate in vitro regeneration techniques are available.

There is general agreement, that by *in vitro* mutation induction techniques, problems associated with the generation of chimaeras can be reduced. Broertjes *et al.* (1976) showed, that plants regenerated via adventitious buds from irradiated *in vivo* leaves of chrysanthemum cv. Bravo were chimaeras in the majority of cases. In contrast, *in-vitro* regeneration of plants from irradiated explants derived from leaves, pedicels or capitula, almost exclusively produced solid non-chimaeral mutants. Histological observations indicated that single epidermal cells were involved in the initiation of adventitious buds in pedicel explants. After X- or gamma-ray treatment of *in-vitro* leaves of the violet-pink flowering chrysanthemum cv. Richmond, a broad variation in flower colour mutants was obtained without any chimaeras (Jerzy, 1990; Jerzy and Zalewska, 1996). In this case also, shoots appeared to originate from single cells of the petiole. Nevertheless, plant regeneration via adventitious buds does not necessarily avoid formation of chimaeras. Whilst Jung-Heiliger and Horn (1980) did not observe any chimaeral flower colour mutants following regeneration of chrysanthemum plants from irradiated cell suspension cultures, chimaeral constitution was often obtained in regenerants from gamma-ray-treated callus cultures. In contrast to these results obtained with chrysanthemum, X- or gamma-ray treatment of *in-vitro*-grown *Gerbera* leaves and subsequent regeneration of adventitious shoots, led to a substantial number of chimaeras within the first vegetative generation (Jerzy and Zalewska, 1996). The proportion of mutants with uniformly altered inflorescences increased considerably in the second vegetative generation, regenerated again from leaf explants. Not surprisingly, the number of chimaeras was high even after two cycles of micropropagation, when plants were regenerated from axillary buds after irradiation of *in-vitro* shoots (Laneri *et al.*, 1990). Among 1250 MV_2 plants, 187 mutants were found, of which only six were assumed to be solid. From 424 variant inflorescences, 35% were chimaeric. In *Weigela*, regeneration of adventitious shoots from ethylmethanesulphonate-treated stem segments rendered both homogeneous mutants as well as chimaeras (Chevreau *et al.*, 1990; Duron, 1992). It must be emphasized, however, that periclinal chimaeras are often advantageous. Certain colour characteristics in petals (e.g. in chrysanthemums) or

in leaves (e.g. bracts of poinsettias) are only realized in chimaeral constitution. Furthermore, mutation induction often results in chromosomal aberration and consequently obtains mutants with both desirable and undesirable traits. In periclinal chimaeras solely possessing a mutated LI-layer, 'negative' alterations of flower size, plant height, yield etc., are not always expressed (Broertjes *et al.*, 1976).

Mutations in Morphology of Flowers and Inflorescences

Recently, several genes responsible for flower morphology have been identified by analysing floral homoeotic mutants mainly in model species such as *Arabidopsis* and *Antirrhinum.* Van der Krol and Vorst (1997) discussed the potentials of genetic engineering for manipulation of flower shape. Such attempts are still at the developmental stage, and it remains to be seen whether it is possible to obtain novel flower shapes of commercial value with justifiable economic costs. In the past, the traditional mutation breeding has considerably contributed to diversity in flower morphology of ornamentals; however, in most cases without elucidating the genetic basis.

Induced mutations affecting the size of flowers or inflorescences have been reported frequently (Table 2). More often, unfavourable reduction in flower size has

Table 2. Induced mutations in size of flowers and inflorescences

Genus	Alterations	Reference
Achimenes	Smaller	B&vH*
Alstroemeria	Smaller	B&vH
Begonia	Larger	B&vH
Brachycome	Smaller	Walther and Sauer, 1989b
Callistephus	Smaller	Wosinska, 1986
Crossandra	Larger	Lalitha *et al.*, 1992
Dahlia	Larger	B&vH
Dendranthema	Smaller or larger	Broertjes *et al.*, 1980; Nagatomi, 1991; MBNL, † 1992; Ahloowalia, 1992
Dianthus	Larger	B&vH
Eustoma	Smaller	Nagatomi *et al.*, 1996
Forsythia	Larger	van de Werken, 1988
Kalanchoe	Larger	B&vH
Ornithogalum	Smaller	B&vH
Pelargonium	Smaller	B&vH
Rhododendron	Smaller	B&vH
Rosa	Smaller	B&vH; Walther and Sauer, 1986b
Streptocarpus	Smaller or larger	B&vH
Weigela	Smaller	Duron and Decourtye, 1986

Induced alterations in flower size were mentioned, but unspecified, for the following species: *Canna*, *Muscari*, *Portulacca*, *Tulipa* (B&vH), *Gerbera* (Walther and Sauer, 1986a) and *Sinningia* (Paek and Han, 1988).

*B&vH = Reports mentioned by Broertjes and van Harten (1988).

†MBNL = *Mutation Breeding Newsletter*, list of cultivars.

been observed. For many species, mutation induction led to changes in flower shape, which were sometimes of ornamental value (Table 3). In a number of species, either an increase or decrease of petal number, or both, were recorded. For example, following mutagenic treatment, several new commercial cultivars of *Begonia elatior* with double flowers were released in the mid-1970s (B&vH). After gamma-ray treatment of *in-vitro* cultured petals of double-flowering carnations, regenerants with double, semi-double or single flowers were found (Simard *et al.*, 1992). Cassells *et al.* (1993) also obtained induced mutants in carnation, producing flowers with only one whorl of petals. Mutants with double flowers were described for *Gladiolus* (Lysikov, 1990) and *Hyacinthus* (B&vH). A single-flowered mutant was obtained from a double-flowered *Hibiscus* and released as a new cultivar (Banerji and Datta, 1986). In contrast to annual *Portulacca*, in perennial genotypes, only double flowers were recorded and mutants with semi-double or single flowers were induced by mutagenic treatment (Datta, 1991a). In *Rosa*, both an increase as well as a reduction in petal number have been obtained through mutation induction (B&vH; Walther and Sauer, 1986b).

For several members of the *Compositae*, induced alterations in 'petal number' or 'number of petal whorls' have been described. In *Chrysanthemum carinatum*, the induction of mutants with single and double type inflorescences has been reported (B&vH). An increase in whorls of ligulate florets was induced in *Gerbera* (Walther and Sauer, 1986a) and *Brachycome* (Walther and Sauer 1989b). Gamma-ray treatment of *Zinnia* seeds produced flower colour mutants that were associated with changes in inflorescence morphology. The number of ray floret whorls was 1–2 in yellow mutants, 2–4 in red mutants with or without white spots, and 5–6 in magenta-coloured mutants (Venkatachalam and Jayabalan, 1994b).

In *Compositae* the conversion of ligulate florets into tubular florets was observed. Following X-ray treatment of *in-vitro* cultured nodal segments of *Brachycome*, Walther and Sauer (1989b) found a mutant with all tubular ray florets, thus displaying the underside of the petals, and giving an impression of almost white-coloured inflorescences. Repeatedly, chrysanthemum mutants with all ligulate flowers turned into tubes have been described (Jerzy, 1990; Datta, 1991a).

Induced mutations in flower morphology have been reported from seed-propagated species as well. In *Petunia nyctaginiflora*, seeds were treated with chemical mutagens and two mutants of putative ornamental value were selected in the M_2 generation (Mahna and Garg, 1989). One genotype produced flowers with broad corollas displaying a dentate margin. Corollas of the other mutant were 'dissected' down to one-third the length of the flower, and the tips of petals were toothed. This trait was monogenic and inherited in a recessive manner. With the aim of generating *Eustoma* genotypes with spray inflorescences, young plants were chronically irradiated followed by *in-vitro* regeneration of shoots from floral petals and leaf blades. After repeated selfing of selected mutants, three stable lines showing spray-type inflorescences with small flowers of funnel- or bell-shaped form, respectively, were selected and submitted for registration as new cultivars (Nagatomi *et al.*, 1996).

Table 3. Induced mutations in shape of flowers and inflorescences

Genus	Alterations	Reference
Begonia	Double flowers, ruffled petals	B&vH*
Brachycome	Broader petals, shorter petals, 'petal whorls doubled', ray florets tubular	Walther and Sauer, 1989b
Chrysanthemum (carinatum)	Double-type inflorescence, single-type inflorescence, ray florets tubular	B&vH
Dahlia	Compact inflorescence, narrow petals, ray florets devided at tip, spider-cactus type	B&vH
Dendranthema	'Petal number', ray florets tubular, inward curved, 'ray-shaped petals'	B&vH; Datta, 1991a; Jerzy, 1990; Ahloowalia, 1992
Dianthus	'Fewer ray florets', single flowers, semi-double flowers	B&vH; Simard *et al.*, 1992; Cassels *et al.*, 1993
Eustoma	Spray-type inflorescence	Nagatomi *et al.*, 1996
Gerbera	Number of ray florets, size of ligules	Walther and Sauer, 1986a; Laneri *et al.*, 1990
Gladiolus	Double flowers	Lysikov, 1990
Hibiscus	Single flowers, semi-double flowers	B&vH; Banerji and Datta, 1986
Hyacinthus	Double flowers	B&vH
Klanchoe	Petals not coalescent, petal margins dissected, petals of round shape	Schwaiger, 1992
Pelargonium	Shape of petals	B&vH
Petunia	Dentate corolla, dissected corolla	Mahna and Garg, 1989
Portulaca	Single flowers, semi-double flowers, '*Gerbera*-type flowers', split petals	B&vH; Datta, 1991a
Rhododendron	Shape of petals	B&vH
Rosa	Reduced petal number, increased petal number, shape of petals, attractive bud form	B&vH; Walther and Sauer, 1986b; Datta, 1989
Rudbeckia	'*Gerbera*-type flowers'	Shukla *et al.*, 1986
Streptocarpus	Picotee, 'Primula-like flowers'	B&vH
Tulipa	Parrot, fringed	B&vH
Zinnia	Ray floret whorls increased	Venkatachalam and Jayabalan, 1994b

Induced alterations in flower shape were mentioned, but unspecified, for the following species: *Canna*, *Muscari*, *Ornithogalum*, (B&vH) and *Antirrhinum* (MBNL,† 1992).

*B&vH = Reports mentioned by Broertjes and van Harten (1988).

†MBNL = *Mutation Breeding Newsletter*, list of cultivars.

The reduction in length of pedicles or peduncles has been reported for some species, such as *Alstroemeria*, *Streptocarpus* or *Tulipa* (B&vH). In *Gerbera*, 42% of mutants displayed a decreased length of the scape (Walther and Sauer, 1986a). A mutant of *Crataegus* with short fruit stalks was selected for putative ornamental use (Li *et al.*, 1993). An increase in length of peduncles was observed in *Dahlias* (B&vH).

Mutations in Leaf Characteristics

Changes in leaf characteristics have been the subject of mutation breeding for improving the balance between leaves and inflorescences, or to alter the leaf coloration including variegation. Induced chlorophyll defects are not the subject of this review unless distinct heritable patterns of 'putative ornamental use' were obtained.

Leaf variegation was achieved in a variety of species (Table 4) and has been exploited commercially, for example in *Abelia*, *Begonia*, *Ficus*, *Guzmania*, *Hoya*, ornamental *Malus*, *Polyanthes*, *Tulipa*, *Weigela* (B&vH) as well as in *Bougainvillea*, *Lantana* (Datta 1991a, 1995) and *Hibiscus* (Maluszynski *et al.*, 1992). Variation in quality or intensity of leaf pigmentation has been induced by mutagenic treatment. A reduction in the chlorophyll content was associated with lighter coloration of flowers in *Weigela* (Duron and Decourtye, 1986) and *Forsythia* (van de Werken, 1988). Mutations that affected the autumn coloration of leaves have been described for shrubs. In *Weigela*, a cultivar with variegated leaves that turn red in the autumn has been marketed (B&vH). A *Forsythia* cultivar with red shoot tips and maroon autumn leaf colour was derived from a selected seedling obtained from a mutated shoot (van de Werken, 1988).

Variation in leaf size as a result of mutation induction has been reported (Table 4) and in some cases generated improved genotypes. One of the spray-type mutants in *Eustomia*, mentioned earlier, developed additionally slender leaves that resulted in an elegant plant habit (Nagatomi *et al.*, 1996). Induced changes in leaf shape and form of leaf margins have often been observed (Table 4). In *Kalanchoe*, for example, regenerated plants from different types of irradiated *in-vitro* explants showed considerable variation in leaf margins ranging from more serrated to almost completely smooth as compared to the original genotype (Schwaiger, 1992). In species with pinnate leaves, the number of leaflets was reduced by mutagenic treatment, e.g. in roses (Walther and Sauer, 1986b). In *Zinnia*, a trifoliate leaf mutant was obtained by Venkatachalam and Jayabalan (1992). Even though induced changes in leaf shape or form of leaf margins had no direct ornamental value, they were used to obtain the plant breeders' rights.

In sterile, double-bracted genotypes of *Bougainvillea*, mutation induction is considered as the only possible method to create genetic variability (Datta, 1992), until genetic transformation methods for this species are developed. Irradiation of stem cuttings was undertaken in India with both single- and double-bracted genotypes. Several mutants with leaf variegation were obtained from different cultivars

Table 4. Induced mutations in leaf characteristics

Genus	Alteration	Reference
Abelia	Variegation	B&vH*
Acalypha	Shape, pigmentation	B&vH
Achimenes	Pigmentation	B&vH
Aechmea	Variegation	B&vH
Anthurium	Size, colour of spathe	B&vH
Begonia	Serrated margin, shape, size, pigmentation, variegation	B&vH; Benetka, 1987
Bougainvillea	Colour of bracts, variegation	B&vH; Deng and Liu, 1990; Datta, 1991a, 1992; MBNL,† 1992
Brachycome	Size	Walther and Sauer, 1989b
Callistephus	Size	Wosinska, 1986
Canna	Variegation	Khalaburdin, 1991
Coleus	Variegation	B&vH
Dahlia	Pigmentation	B&vH
Dendranthema	Shape, size	Jung-Heiliger and Horn, 1980; Nagatomi, 1991; Ahloowalia, 1992
Dianthus	Shape	Cassells *et al.*, 1993
Euphorbia (pulcherrima)	Shape, size, colour, colour of bracts, shiny bracts, size of bracts	B&vH, personal communication by German breeders
Eustoma	Size	Nagatomi *et al.*, 1996
Ficus	Variegation	B&vH
Forsythia	Shape, size, autumn colour	B&vH, Van de Werken, 1988
Gerbera	Shape, size	Walther and Sauer, 1986a
Guzmania	Variegation	B&vH
Hedera	Variegation	B&vH
Hibiscus	Variegation	MBNL, 1992
Hoya	Shape, pigmentation, variegation	B&vH
Kalanchoe	Shape, size, colour, margins	B&vH; Schwaiger, 1992
Kohleria	Size	Geier, 1988
Lantana	Variegation	Datta, 1991a, 1995
Lilium	Pigmentation, thickness	Wang *et al.*, 1989
Lonicera	Shape, size, colour, variegation	Cambecèdes *et al.*, 1992
Malus	Variegation	B&vH
Muscari	n.d.	B&vH
Passiflora	Shape, pigmentation	Park and Andersen, 1990
Pelargonium	Shape, pigmentation	Grunewaldt, 1983
Petunia	Size, leathery, colour, variegation	Mahna and Grag, 1989
Polyanthes	Variegation	Datta, 1991a
Rhododendron	Shape	B&vH
Rosa	Shape, size	Walther and Sauer, 1986b
Saintpaulia	Pigmentation, variegation	B&vH; Grunewaldt, 1983, 1988
Sansevieria	Variegation	B&vH
Streptocarpus	Size	B&vH
Tulipa	Variegation	B&vH
Weigela	Shape, size, variegation, autumn colour	B&vH; Duron and Decourtye, 1986; Duron, 1992
Zinnia	Shape, chlorophyll	Venkatachalam and Jayabalan, 1992

*B&vH = Reports cited by Broertjes and van Harten (1988).

†MBNL = *Mutation Breeding Newsletter*, list of cultivars.

in both groups of plants and have been marketed as novelties. Induced changes in bract colour have also been observed, but they pre-existed as a result of natural sporting (Datta, 1991a, 1992). Deng and Liu (1990) reported changes in bract colour in the range of 0.74–1.64% after treating rooted cuttings with 30 or 35 Gy gamma-rays.

In *Euphorbia pulcherrima*, the predominant share of marketed plants is composed of red-bracted cultivars, supplemented by genotypes with pink, variegated ('marble type') and white bracts in the range of 5–20%, depending on regional preferences. Bract colour is determined by the action of two genes and, additionally, depends on the cell layer composition of a given genotype. Anthocyanin synthesis leading to red bracts results from the expression of a single dominant gene *WH*, while homozygous recessive genotypes have white bracts (Stewart, 1960). The second gene (*pk*), involved in homozygous recessive state, reduces the quantity of anthocyanin synthesis, thus resulting in pink bracts (Stewart and Arisumi, 1966). Additionally, periclinal chimaeras lacking the ability to produce anthocyanins within the LI-layer, also carry pink bracts (Bergann, 1961). The possible types of chimaeras and homohistonts leading to red, pink, marble or white bracts are summarized in Figure 3, without consideration of the *pk* gene that contributes additional variation. According to this situation *Euphorbia pulcherrima* has been an interesting object for mutation induction in both scientific research and commercial plant breeding. The occurrence of spontaneous mutants and subsequent cell layer rearrangements resulting in sport families, as well as the segregation of apical chimaeras by in vitro culture techniques have been discussed by Preil (1986). The *WH* gene for anthocyanin synthesis has been used as a marker gene in heterozygous genotypes to optimize parameters for *in-vitro* mutagenesis. Induced homozygous *whwh* mutants are readily identified by anthocyanin-free petioles, even during *in-vitro* culture (Kleffel *et al.*, 1986). The same trait is used for early selection of mutants in the greenhouse by commercial plant breeders. The *Whwh* genotype is most promising for inducing the whole spectrum of colour alterations. Even white-bracted mutants have been repeatedly found in the M_1 generation. In addition to the reduction in anthocyanin content, genotypes with a more intense red colour as well as types with a more shiny surface have been found. As for other plant species, selected genotypes have to be evaluated carefully because there may have been more than one mutational event. For example, changes in photoperiodic response and an increase in the duration required for flower development have been associated with some promising mutants of several cultivars. Although, mutation induction is now routinely applied and integrated in breeding of *Euphorbia pulcherrima*, no cultivar has been officially announced as being a selected mutant.

Mutations in Growth Habit Characteristics

The objective of many breeding programmes is to develop compact plant types with good branching in flowering or foliage pot plants and in woody ornamentals.

colour of bracts	cell layer	anthocyanin synthesis	genetic constitution
red	L1	yes	*WH WH* or *WH wh*
	L2	yes	*WH WH* or *WH wh*
	L3	yes	*WH WH* or *WH wh*
pink	L1	no	*wh wh*
	L2	yes	*WH WH* or *WH wh*
	L3	yes	*WH WH* or *WH wh*
marble	L1	no	*wh wh*
	L2	no	*wh wh*
	L3	yes	*WH WH* or *WH wh*
white	L1	no	*wh wh*
	L2	no	*wh wh*
	L3	no	*wh wh*

Figure 3. Main types of periclinal chimaeras and homohistonts in *Euphorbia pulcherrima* leading to the development of red, pink, marble or white bracts, respectively

In addition, several dwarf garden plants are now being marketed as pot plants. The excessive application of growth-retarding chemicals is not tenable for ecological and economic reasons, and is no longer accepted by the public. Therefore, different strategies – including mutation induction – are being pursued in order to broaden the genetic variability, and to select dwarf genotypes amenable for pot culture.

Induced alterations in plant habit have been reported in many plant species following the mutagenic treatment. Mostly, compact and dwarf forms have been mentioned in the literature (Table 5); however, in many cases, no information is available on the stability and heritability of the observed traits. In contrast to mutational events expression of primary radiation damages usually disappears during vegetative propagation.

Table 5. Induced mutations in growth habit characteristics

Genus	Observed alterations	Reference
Achimenes	Higher, vigorous, compact	B&vH*
Anthurium	Dwarf	B&vH
Begonia	Dwarf, compact, upright, vigorous	B&vH; Benetka, 1987
Bidens	Compact	Personal communication by German breeders
Brachycome	Dwarf	Walther and Sauer, 1989b
Callistephus	Compact, shorter shoots	Wosinska, 1986
Canna	Dwarf	Khalaburdin, 1991
Chamaecyparis	Juvenile habit	B&vH
Coix lacryma-jobi	Semi-dwarfness	MBNL,† 1996
Coleus	Dwarf	B&vH
Dahlia	Height	B&vH
Dendranthema	Height, compact	Broertjes *et al.*, 1980; Ahloowalia, 1992; Tulmann Neto and Latado, 1996
Dianthus	Dwarf	MBNL, 1992
Euphorbia	Height, compact, dwarf	B&vH; personal communication by German breeders
Ficus	Fast-growing, compact	B&vH
Forsythia	Compact, dwarf, semi-dwarf, larger	B&vH; Van de Werken, 1988
Gerbera	Size	Walther and Sauer, 1986a
Gladiolus	Very short stems, better corm production	B&vH; Lysikov, 1990
Impatiens	Dwarf	B&vH
Jasminum	Dwarf	B&vH
Kalanchoe	Dwarf, compact, branching habit, upright	B&vH; Schwaiger, 1992
Kohleria	Compact	Geier, 1989
Lantana	Branching habit	Datta, 1991a
Lonicera	Compact, slender	Cambecèdes *et al.*, 1992
Ornithogalum	Smaller, larger	B&vH
Petunia	Dwarf, bushy	Mahna and Garg, 1989
Rhododendron	Dwarf	MBNL, 1992
Rosa	Climbing types, compact	B&vH; Walther and Sauer, 1986b
Streptocarpus	Compact, dwarf, finer habit, more sturdy, plant architecture	B&vH; MBNL, 1992
Weigela	Soil covering type, compact, vigour	B&vH; Duron and Decourtye, 1986; Duron, 1992
Zinnia	Dichotomous branching	Venkatachalam and Jayabalan, 1991, 1992

Induced alterations in growth habit were mentioned, but unspecified, for the following species: *Muscari* (B&vH), *Passiflora* (Park and Andersen, 1990) and *Portulaca* (Abo-Hegazi, 1991).

*B&vH = Reports mentioned by Broertjes and van Harten (1988).

†MBNL = *Mutation Breeding Newsletter*, list of cultivars.

In a few cases induced changes in branching habit have been observed. In *Zinnia*, a conversion to dichotomous branching was found in yellow and red-flowered colour mutants (Venkatachalam and Jayabalan, 1991). In *Lantana depressa*, as late as in the M_1V_5-generation three mutants developed three axillary branches at each node instead of having normally two (Datta, 1991a, 1995). In *Kalanchoe*, a compact branching mutant has been isolated and marketed as new cultivar Flores (B&vH).

The reduction in plant height is caused by decrease in the number of internodes, or internode length or both. The inheritance of dwarf characteristics can either be dominant or recessive or intermediate. For example, the dwarfness is controlled by a single dominant gene in *Rosa chinensis minima* (Dubois and De Vries, 1987) and by one recessive gene in *Impatiens platypetala* (Weigle and Butler, 1983). The genetic background of dwarfness not only varies between species, but even within the same crop (Gottschalk and Wolff, 1983). The reduction in plant height can be classified into three groups: (1) stunted dwarf, (2) dwarf, and (3) semi-dwarf. The classification of height within dwarf phenotypes differs between species. For example, *Kohleria* mutants were described as: 'stunted dwarf' with rosette growth up to 25%, 'dwarf' ranging from 25% to 50%, and 'semi-dwarf' from 50% to 75% of the original plant height (Parliman and Stushnoff, 1979).

Compact and/or dwarf types of putative ornamental value have been found in several herbaceous species after mutagenic treatment (Table 5). Walther and Sauer (1989b) described two induced dwarf mutants of *Brachycome*. One of them is a cushion-type plant; the second dwarf mutant is an early and more free-flowering type. *N*-nitroso-*N*-methylurea treatment of *in-vitro* internodes of a sterile hybrid of *Kohleria* gave rise to considerable variation in plant height and internode length (Geier, 1988). This approach enabled selection of genotypes with a 50% reduction in height and internode length, suitable as pot plants without applying growth retardants. Dwarf *Saintpaulia* have been obtained by mutagenic treatment (Jungnickel, 1977). While studying a range of *Kalanchoe* cultivars, Schwaiger (1992) found several compact mutants with shortened internodes and peduncles, some of which were of horticultural interest. Plant habit of *Kalanchoe* was also affected by somaclonal variation (Schneider-Moldrickx and Horn, 1985; Schwaiger and Horn, 1988) and was demonstrated to be the trait most frequently altered (Schwaiger, 1992). By irradiation treatment of *Petunia nyctaginiflora* seeds, a dwarf mutant with erect and bushy habit was found in the M_2 generation. Few sterile flowers were produced by this mutant, and this precluded further exploitation in breeding (Mahna and Garg, 1989).

Rosa is one of the species among several woody ornamentals evaluated for dwarf and compact types after mutagenic treatment. *In-vitro*-grown shoots of cv. Ilseta were irradiated and micropropagated via axillary buds, and results showed that 14% of regenerants expressed alterations in growth habit. Various dwarf types were selected, including bushy plants with shortened branches. Some of them had three to five flowers blooming simultaneously and were considered suitable as pot plants. Conversely, some mutants with elongated internodes were found and classified as 'creepers' (Walther and Sauer, 1986b). Also, in *Rosa*,

climbing types frequently resulted from mutation induction (B&vH). In *Weigela*, irradiation or ethylmethanesulphonate treatment of *in-vitro* shoots or explants resulted in mutants with altered vigour and growth characteristics (Duron and Decourtye, 1986; Duron, 1992). One interesting mutant to be used as 'ground cover', has been selected (Duron and Decourtye, 1986). In *Lonicera*, *in-vitro* mutagenesis was specifically aimed at selecting plants with reduced vigour and shortened internodes. Microcuttings of five species were treated with gamma-rays and subcultured three times via axillary buds. Subsequent evaluation of regenerated plants was restricted to 200 plants derived from cv. Maigrün. Still, nine mutants were selected, including such with compact or slender growth habit (Cambecèdes *et al.*, 1992). Among five new released cultivars of *Forsythia*, obtained either by direct selection or as seedlings from mutated shoots, three of them differed in growth pattern: (1) semi-dwarf 'Tinkle-Bells', (2) dwarf 'Minikin' with very short internodes, and (3) large 'Fairy-Land' with more rapid growth (Van de Werken, 1988). In *Passiflora*, mutagenic treatment also changed growth habit (Park and Andersen, 1990).

Many vigorously growing free-flowering tropical shrubs represent a valuable source for putative 'new' ornamentals. Mutation breeding methods are appropriate for the induction and selection of dwarf mutants suitable as pot plants. Recurrent irradiation may increase the efficiency of mutant selection, as demonstrated in *Rhododendron simsii* by De Loose (1979). With three consecutive treatments during three years, 31% of plants had chimaeric flowers. *In vitro* mutagenesis will speed up the procedure in those species which can be easily micropropagated. A selection scheme has been developed for tetraploid sterile *Tibouchina urvilleana*, which was expected to represent the quadruplex genotype AAAA for plant height (Fig. 4; Preil *et al.*, in preparation). The internode length of this genotype exceeds 20 cm under optimum growth conditions. During an 18-month period, nodal segments were cut from *in-vitro*-cultivated plants and were recurrently X-ray treated by using a split-dose system (3×15 Gy) to obtain dwarf mutants of hypothetic nulliplex genotype aaaa. After the third, fourth and fifth irradiation, one part of the regenerated plants was transferred to the greenhouse for mutant screening, while the other part remained under *in-vitro* culture conditions to be used for the next mutagenic treatment. Out of 3100 regenerated plants after the third X-ray treatment, only two dwarfs (0.06%) were found. After the fourth irradiation, 6200 plants were screened, and 51 dwarfs (0.82%) were selected. The percentage of dwarfs increased to 1.12% (65 dwarfs among 5800 plants) after the fifth irradiation (Figs 4 and 5). The solid mutant character or chimaeral nature of selected dwarfs has not yet been confirmed.

Physiological Mutants

The genetic basis of most physiological traits is poorly understood. In general, more than one gene is involved in the expression of each complex trait, e.g. induction of the generative phase or tolerance against environmental stress. Only

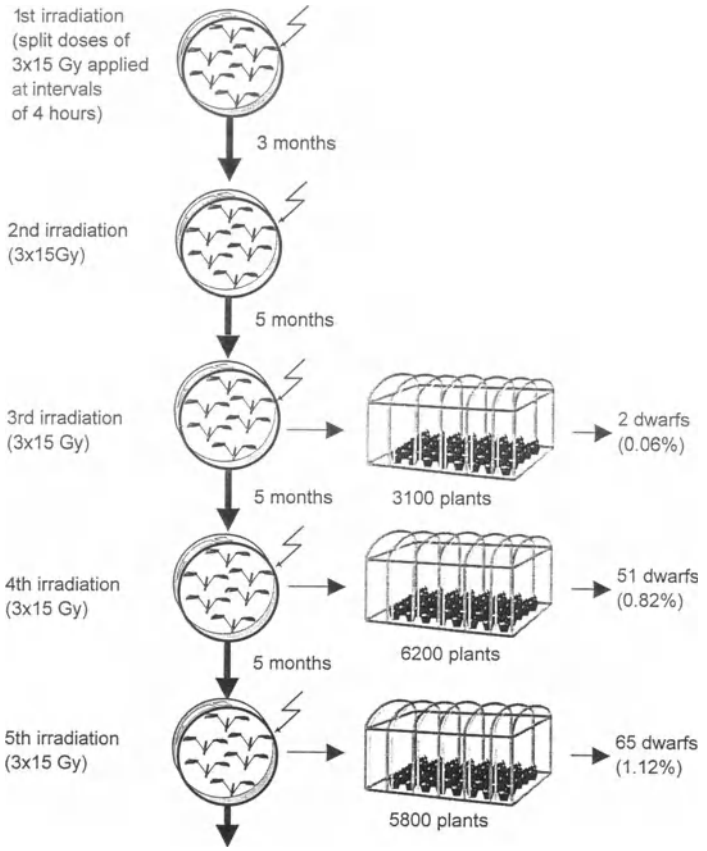

Figure 4. Scheme for induction and selection of dwarf tetraploid *Tibouchina urvilleana* using recurrent X-ray treatment of *in-vitro*-cultured nodal segments

a few papers report on simple Mendelian segregation. In *Pelargonium*, early flowering is inherited by one dominant gene (Hanniford and Craig 1982). A single recessive gene, when present in its homozygous state, controls recurrent flowering in rose (Semeniuk 1971, De Vries and Dubois 1978). In mutation breeding however, direct selection methods do not necessarily require information on the number of genes involved, because many of the polygenic characters can be altered significantly by mutation, where only one of the loci is affected. An overview on selection of the physiological traits in ornamental plant breeding has been published by De Jong (1991).

Low temperature tolerance

The increase in energy prices influenced the production costs of glasshouse-grown ornamentals and raised interest in low-temperature-tolerant cultivars. The first report on selection of cold-tolerant variants from irradiated unrooted cuttings of

Figure 5. Tibouchina urvilleana: shoots of the original genotype (left) and a dwarf mutant (right)

chrysanthemum was by Jung-Heiliger and Horn (1980). Selected clones flowered in a temperature regime of 12/20°C (night/day) at the same time as at 16/20°C, without a loss in flower quality. Similar results were obtained by Broertjes *et al.* (1983) who irradiated rooted cuttings of several cultivars. The selection at low temperatures (15–16°C, instead of 17–20°C as is normal) resulted in a relatively high percentage (~1%) of low-temperature-tolerant mutants (LTT-mutants). However, some years later these LTT-mutants had lost their tolerance, most probably because they were periclinal chimeras and reverted unnoticed to the original genotype (Broertjes and Lock 1985). Such unfavourable reversions can be avoided if solid non-chimaeric mutants are produced either from irradiated cell suspension cultures or from explants regenerating adventitious shoots from single cells. Jung-Heiliger and Horn (1980) reported no chimaeras in progenies derived from mutagen-treated cell suspension cultures, in contrast to clones from irradiated cuttings which resulted in a high frequency of chimaeras. However, genetically stable winter-flowering mutants were induced by gamma-irradiation of chrysanthemum cuttings (Hu *et al.*, 1989) and in selected clones flowering was initiated below 9°C.

Preil *et al.* (1983) reported for the first time chrysanthemum LTT mutants, selected from *in-vitro* cell suspension cultures at low temperature. Plated cells of X-ray-treated suspension cultures of the pot-grown cultivar 'Puck' were exposed to 8°C for 170 days. Subsequently, surviving cell aggregates regenerated shoots at 24°C. Approximately 2000 plantlets were grown during the winter at 10°C in the greenhouse. Only 16 plants that were able to induce flowers at 10°C were recognized as solid LTT mutants. All other regenerated plants were epigenetic variants that lost low-temperature tolerance during greenhouse culture. Further investigations showed that the cold tolerance of the LTT mutants was expressed solely in the generative phase resulting in early flowering. This trait was strictly associated with low-temperature stress and did not express during summer at high temperature (Preil *et al.*, 1988). In another set of experiments with cv. Puck and two of its LTT mutants, it was found that at low night temperature mutants produced less leaves before flowering thus indicating a more efficient metabolism for flower set (Zimmer and Preil, 1987).

Broertjes and Lock (1985) obtained solid, non-chimaeral mutants when using irradiated explants of chrysanthemum pedicels for regeneration of adventitious shoots. During direct selection of regenerants in cv. Parliament at 12°C (greenhouse temperature regime), 3.6% of plants were LTT mutants. Some of them flowered 2 weeks earlier than the parental cultivar.

Based on the results obtained with direct LTT mutant screening in the greenhouse at low temperature (Broertjes *et al.*, 1983; Broertjes and Lock, 1985) and with preselection at the cellular level (Preil *et al.*, 1983), respectively, a series of experiments was carried out in order to determine the best technique for practical breeding (Huitema *et al.*, 1986, 1987, 1989, 1991; De Jong *et al.*, 1987, 1991; Preil *et al.*, 1991). Cell suspension cultures of chrysanthemum cv. Parliament were treated with X-rays and subjected to either 6°C stress temperature for 90 days or immediately cultured at 24°C for shoot regeneration. The evaluation of plants gave LTT mutants from both the treatments (Huitema *et al.*, 1989, 1991; Preil *et al.*, 1991). However, no advantage of *in-vitro* preselection at low temperature could be seen, possibly because of underestimated negative influences of phenolic compounds released by the large number of dying cells during low-temperature treatment. Phenolic compounds are assumed to be toxic to adjacent mutant cells, which otherwise are thought to be less inhibited by temperature stress. This might be the cause for lacking accumulation of LTT mutants within the surviving population. The only way to avoid the negative effects of released phenolic compounds is to separate the growing calli from dead aggregates by subculture on fresh medium. Such labour-intensive subculturing would need to be repeated several times, thus diminishing the putative advantage of *in-vitro* selection. Most LTT mutants originated from direct screening in the greenhouse at 12°C and were predominantly derived from slowly regenerating calli. This is perhaps due to the fact that 'fast' regenerating calli were mostly unaffected by the mutagenic treatment, while 'slow' regenerating calli were partly damaged by irradiation. DNA repair mechanisms induced after irradiation could have delayed shoot development.

X-ray treatment of chrysanthemum cell suspension cultures was more efficient with 15 Gy than with 20 Gy, which caused more cell damage. Somaclonal varia-

tion in the unirradiated control cultures was ineffective to select LTT mutants. This contrasts with the results of Benetka and Kodytek (1988), who found that somaclonal variation could give rise to a high percentage of LTT mutants (60% in cv. Delta). In their experiment with nine cultivars gamma-irradiation (8–15 Gy) did not significantly increase the number of LTT mutants in most cases. The large range of genes controlling low-temperature tolerance in chrysanthemum cultivars may explain these extremely divergent observations.

A similar screening procedure at cellular level, as decribed for chrysanthemum cv. Puck (Preil *et al.*, 1983), was applied to cell suspension cultures of poinsettia (*Euphorbia pulcherrima*) cv. Preduza. A total of 977 plants were regenerated from 25 Gy-irradiated cells, which were exposed *in vitro* to low temperature at 12°C for 170 days. Only four mutants were isolated that were slightly more adapted to 14°C in the greenhouse than the original genotype. The improvement, however, was too small from the practical point of view, although the parameter measured (e.g. reduced leaf abortion) differed significantly ($p = 5\%$) from the control. One of the LTT mutants also showed faster bract development, being fully expanded one week earlier than the original genotype. So far, no further published information is available on the selection for cold-tolerance at the cellular level in ornamental species.

Limited information is available on LTT mutants in ornamentals, selected from tissue and organ cultures other than in chrysanthemum and poinsettia. In *Saintpaulia*, attempts were made to select LTT genotypes (Amberger *et al.*, 1984; Grunewaldt, 1988) with or without mutagenic treatment. However, the subsequent impact on horticultural practices was not reported. In *Fuchsia*, selection for LTT lines from callus cultured at 13°C in the dark has been attempted, although further details are lacking (Bouharmont and Dabin, 1986). Improved winter hardiness of *Iris* hybrids was induced by gamma-irradiation of seeds (Dryagina, 1989), leading to the release of 13 cultivars of good ornamental quality.

Low Light Tolerance

Low light intensity usually causes growth depression, inhibition of flower initiation or flower bud abortion, leading to an overall reduction in plant quality. During winter, in Northern countries, light intensity becomes a limiting factor in greenhouse plant production. Since the 1970s, breeding and selection programmes for low-light tolerance have been reported, e.g. in chrysanthemum (Satory, 1977; De Jong, 1986, 1989) and *Lilium* (van Groenestijn and van Tuyl, 1983; van Tuyl *et al.*, 1985).

A LTT chrysanthemum mutant of cv. 'Puck' (Preil *et al.*, 1983) was less sensitive to low-light conditions as compared to the original genotype (Zimmer and Preil, 1987). At 18/17°C (day/night), cv. 'Puck' started flowering after 61 days under high-light conditions, whereas under low-light condition flowering began after 73 days. In contrast, mutant 82/37-1 developed flowers after 54 and 59 days, respectively. At 11°C night temperature, cv. 'Puck' flowered under high-light after 65 days and under low-light after 78 days. Mutant 82/37-1 required 58 and 65 days, respectively.

In *Lilium* 'Enchantment' and 'Connecticut King', low-light tolerant mutants were obtained. The flower bud abscission was either absent or reduced during winter culture without supplementary illumination (van Groenestijn and van Tuyl, 1983). From one clone, van Tuyl *et al.* (1985) obtained different mutants with various responses to light intensity. They expected that the selection would yield commercial cultivars with an acceptable performance at a lower light intensity.

Mutants of a sterile *Kohleria* hybrid ((*Kohleria amabilis* × *K. bogotensis*) × *K. eriantha*) were induced by *N*-nitroso-*N*-methylurea-treatment (Geier, 1988, 1989, 1994). The parental type has attractive flowers but long internodes, large leaves and is late flowering under low-light conditions. One mutant was selected which is early flowering under low-light conditions. In addition, the mutant has much shorter internodes and smaller leaves. These characters remained stable after vegetative propagation, thus confirming the mutant nature.

Broertjes and van Harten (1988) listed numerous mutants of *Alstroemeria*. Two of them, 'Atlas' and 'Pink Tiger', were characterized as highly suited for winter production compared with the original cultivar. A mutant of *Streptocarpus* cv. Constant Nymph with a drastic change in its flowering behaviour was induced by X-irradiation (Davies and Hedley, 1975). Whereas the original genotype flowered under long-day conditions, the mutant was able to set flowers under both long- and short-day regimes. It was speculated that the difference in the behaviour of these two types may not necessarily be due to phytochrome activities. It could be equally related to the differences in photosynthetic activities or in partitioning of the assimilates between the normal and mutant genotypes.

Salt Tolerance

There are many publications on efforts to select salt-tolerant lines, especially in agronomically important species. Gradual adaptation of cultured cells or organs to salt tolerance has often been reported, which was obviously epigenetically controlled. In other cases, the stability or heritability of the selected salt-tolerant plants were not demonstrated nor discussed. Only two reports are available on salt tolerance in ornamental plants. Plantlets were regenerated from ethylmethanesulphonate-treated chrysanthemum callus that survived on the selection medium supplemented with 10 g NaCl/L (Dalsou and Short, 1987). The regenerants accumulated greater amounts of sodium chloride than the control plants. Furthermore, the selected variants were more succulent than the control plants, but exhibited comparable growth characteristics. Details to confirm the mutant status of the selected plants were not given. In *Coleus blumei*, somaclonal variation led to the selection of salt-tolerant plants (Ibrahim *et al.*, 1992). Leaf disc explants were exposed to 90 mM NaCl added to MS basal medium. The regenerated plantlets were grown to maturity and compared with the control plants. The cuttings from selected salt-tolerant plants, as well as their seed progeny, showed consistently greater growth than the control, both in the presence and absence of NaCl. These results suggested that *in-vitro* selection favoured vegetative vigour rather than specific enhancement of salt tolerance.

Disease Resistance

Problems associated with ornamental plant breeding for disease resistance which also affect strategies for mutation induction were discussed by Sparnaaij (1991). *In vitro* culture methods, so far mainly applied to agronomically important species, offer efficient support to breeding activities (Jones 1990). The main problems of induction and selection of resistant mutants arise from low mutation rates of relevant genes and from difficulties in analyzing the mutant characters, for example, degree of resistance, stability and heritability of altered traits, and chimaeral constitution of regenerants.

In carnation, two mutants ('Maiella-lonchabi' and 'Galatee-lonvego') selected from gamma-irradiated cuttings were designated as 'more resistant' to *Fusarium oxysporum* f.sp. *dianthi* (Mitteau and Silvy, 1982). Another mutant ('Loncerda') proved to be 'very resistant' to *Fusarium* (Mitteau and Silvy, 1983). The details were not given on specific resistance to one or more of the four races known in *Fusarium oxysporum* f.sp. *dianthi* (Sparnaaij, 1991). Baayen *et al.* (1991) showed that resistance to race 1 appeared to be under monogenic control, whereas resistance to race 4 was polygenically inherited. This may complicate the characterization of induced mutants.

Efforts in selecting mutants from X-irradiated nodes of carnation cv. Mystère gave rise to lines with increased resistance to *Alternaria* disease (Cassells *et al.* 1993). In *Begonia elatior* hybrids cv. Schwabenland Pink, a mutant (cv. Heirloom) with increased resistance to mildew was induced by fast neutrons (Mikkelsen, 1975). The rose mutant 'Pink Hat', was almost completely resistant to mildew; it originated from gamma-irradiation of terminal buds of an unnamed floribunda rose line (James, 1983). Leafspot-resistant mutants of *Jasminum grandiflorum* were induced by Nambisan *et al.* (1980). Furthermore, disease-resistant mutants were reported for *Iris* (Dryagina, 1989), tulips (Kudrjavceva, 1993), and *Ficus benjamina exotica* (De Loose, 1986).

Induced Mutations versus Somaclonal Variation

The problems of somaclonal variation (SV) in ornamentals were recently reviewed by Bouman and De Klerk (1997). The extent of SV is usually determined as the percentage of plants showing aberrations for one or more defined characteristics. It usually varies over a wide range of traits depending on the instability of the genome and the specific *in-vitro* culture conditions (De Klerk, 1990). In some species, SV occurs in high frequencies, for example up to 80% in rice (Buiatti and Gimelli, 1993). The comparison of mutations obtained as a result of tissue culture effects in contrast to chemical mutagenesis in tomato (Gavazzi *et al.*, 1987) or treatment with gamma-rays in maize (Novak *et al.*, 1988), indicated a higher frequency of mutants due to SV as well as a somewhat different mutation spectrum. However, from the data given it remains unclear, whether optimal procedures for mutation induction were ascertained. (Walther and Sauer, 1991).

While comparing the frequency of induced and spontaneous mutants in X-ray-irradiated (15 and 20 Gy) and non-irradiated cell suspension cultures of chrysanthemum cv. Parliament, a relatively low number of spontaneous mutants (somaclonal variants) were found. In total, less than 2% of plants from untreated cultures deviated in flower colour (0.7%), flower size (0.6%) or leaf colour (0.5%). Of plants derived from both irradiated populations, 4.6% showed changes in flower colour. Similar numbers of mutants with small flowers (7.7% and 8.6%) were induced by the 15 Gy and 20 Gy treatments (Table 6). In this case, the results demonstrated the higher efficiency of mutagenic treatment over somaclonal variation. However, a high number (10.4%) of undesired mutants with pale green leaves were regenerated from the 20 Gy-treated cultures.

In many cases, undesirable genetic variability was recovered together with the improvement of desired traits (Buiatti and Gimelli, 1993). A serious drawback of exploiting SV, as a necessary prerequisite, is the availability of an appropriate *in-vitro* culture regeneration system suitable for a wide range of genotypes. Maluszynski *et al.* (1995) concluded that while a great number of radiation-induced mutants have been released as new cultivars, somaclonal variants so far have been of limited value in plant improvement. Bouman and De Klerk (1997) also stressed the limited use of SV in breeding. Buiatti and Gimelli (1993) listed a small number of cultivars known to have been derived from SV in ornamentals.

Mutagenesis versus Molecular Techniques

Mutation induction and genetic engineering of plants aim at modifying single traits by conserving the original genome of elite genotypes. The selection of induced mutants is generally based on the 'useful genotype'. Very little information is available on the molecular changes leading to the 'useful phenotype' (Micke, 1991). Genetic transformation of plants requires profound molecular insight and allows a directed approach whenever a gene encoding a desired trait is available. Furthermore, creating variation by mutation induction is restricted to

Table 6. Comparison of number of induced mutants and somaclonal variants in chrysanthemum cv. Parliament regenerated from X-ray-irradiated and non-irradiated cell suspension cultures

Irradiation dose (Gy)	Number of plants investigated	Mutants			
		Total (%)	Flower colour changes (%)	Small flowers (%)	Pale green leaves (%)
0	875	1.8*	0.7*	0.6*	0.5*
15	1080	14.9	4.6	7.7	2.6
20	280	23.6	4.6	8.6	10.4

*Somaclonal variants. (data from Preil *et al.*, 1991)

the given genetic set-up of plants, whereas molecular techniques allow the introduction of foreign DNA from any source. Thus, genetic engineering will lead to the incorporation of new traits, which are naturally unavailable within the gene pool of the species in question. For example introduction of blue flower colour, which is missing in some of the most important ornamentals such as roses, carnations, chrysanthemums and gerberas may become feasible. In *Petunia hybrida*, plants carrying a new flower colour (orange) have been obtained by genetic transformation and successive sexual recombination (Oud *et al.*, 1995).

Successful transformation protocols are being published for a rapidly increasing number of ornamentals. Recent reviews have listed 22 species indicating production of transgenic tissues (Deroles *et al.*, 1997), and 12 species in which stable transgenic plants have been obtained (Burchi *et al.*, 1996). Strategies for the improvement of ornamentals by genetic transformation have been discussed for traits such as flower colour (Davies and Schwinn, 1997), flower shape (van der Krol and Vorst, 1997) and plant habit (McCown, 1997). As has been pointed out for induced mutants vegetatively propagated ornamentals are especially amenable to genetic engineering. Transgenic plants of interest are often easily micropropagated for further evaluation and ultimate release of cultivars (Hutchinson *et al.*, 1992). At present, however, one of the main bottlenecks of genetic engineering is the lack of protocols applicable to a wide range of genotypes.

Although, genetic engineering of ornamentals has made considerable progress, it is still far from being a routine method that can be universally applied. Major restrictions are related to the availability of genes, difficulties in handling polygenically inherited traits and long-term stability of transgenic plants. Both in induced mutants as well as in transgenic plants instability of the newly acquired characters have been observed. In mutants, this phenomenon has been attributed either to chimaeral constitution or to an undefined mechanism of 'instability of genes'. Instability of transformants has been investigated more thoroughly, and in some cases gene silencing has been associated with an increase in DNA methylation, for example, due to integration of multiple copies at the site of integration (Meyer, 1995). Still, there is a limitation on site-directed targeting and control of single-copy insertion in plants.

The number of plant species cultivated as ornamentals is estimated to be more than 1000 (Burchi *et al.*, 1996). Each one is of minor economic importance as compared to the main agricultural crops. Furthermore, consumer's demand for new products results in a fast turnover of varieties, and sometimes this results in short marketing periods for new cultivars. The investment in sophisticated and expensive breeding methods does not seem adequate in such cases (Burchi *et al.*, 1996). Therefore, recent genetic transformation activities are increasingly focused on the economically important ornamental species such as roses, chrysanthemums and carnations for introducing genes for resistance against insects and various diseases (De Jong *et al.*, 1995; Daub *et al.*, 1997, Löffler and Florack, 1997). However, there are still problems in commercial exploitation of genetic transformation; therefore mutation induction continues to be an attrac-

tive approach for creating genetic variation in ornamentals whenever the desired traits can be expected within the genetic scope of a given species.

Conclusion

Mutation induction has become a routine technique in vegetatively propagated ornamental plants used by commercial breeding companies during the past two decades. In most cases the commercialization of new mutants is no longer announced. Therefore, the number of officially registered mutants does not reflect the real impact of mutation breeding. The scientific interest in mutation research has declined in Western countries during the past 10 years, whereas it seems to continue in Eastern Europe and Asia.

There has been a revival of interest in mutation research during the late 1970s to mid-1980s because of the availability of *in-vitro* regeneration systems in an increasing number of plant species. The advantages of *in-vitro* mutagenesis in comparison with *in-vitro* approaches have often been outlined (Broertjes, 1982; IAEA, 1986; Broertjes and van Harten, 1988), and have become well-established facts. Nowadays, such techniques are included in commercial breeding whenever expected results justify the high overhead costs for the maintenance of *in-vitro* cultures and the manpower costs of highly specialized technicians.

Currently, European scientific activities on mutation breeding are minimal. This is mostly due to limited funding resources that are being increasingly directed to molecular biology. In the near future, genetic engineering may become an established additional tool for ornamental breeders. Nevertheless, mutation induction will continue to contribute to creation of commercial novelties due to its easy and efficient technology.

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18. *In-vitro*-Induced Mutations for Disease Resistance

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Introduction

Spontaneous mutations form the basis of the variation used by plant breeders for plant improvement. Induced mutants supplement natural gene pools. Mutation induction has the advantage that the target genotype is changed only slightly, relative to the genotype derived by crossing of two varieties, and thus the time required to develop a new variety is considerably reduced. Advances in plant cell, tissue and organ culture have provided new opportunities for the production of 'solid' mutants, i.e. homohistonts (Broertjes and van Harten, 1988) and, potentially, for the preliminary screening of large numbers of mutants (Dix, 1990). Historically, mutagenic treatments of bulky vegetative propagules have presented serious limitations regarding the numbers that could be treated. Furthermore, bearing in mind that the frequency of useful mutants is low, the treated material has presented problems for the evaluator. The latter are compounded by the frequent occurrence of chimaeral progeny which show instability of the characters (Broertjes and van Harten, 1988). Mutagenesis of *in-vitro* propagules avoids the need for large-scale facilities and also allows better control of the treatment, as 'vitrified' tissue (Ziv, 1991) may be more permeable to mutagens.

Impetus for mutation breeding has also come from the discovery that spontaneous mutation can arise *in vitro* with a high frequency in cells and may be expressed in the adventitiously regenerated plants ('somaclonal variation'; see reviews by Karp, 1991, 1995). The occurrence of somaclonal variation stimulated attempts to simplify selection procedures, conventionally carried out in the field, by employing preliminary selection at the *in-vitro* level (Dix, 1990), albeit field trials are still needed. The number of publications on somaclonal variation for disease resistance has been about 200 per annum for the past 10 years. In this respect, it is important to recognize that tissue culturists deal mainly with vegetatively propagated crops, many of which are infertile or have highly heterozygous polyploid genomes that are not amenable to conventional breeding. While the agronomic characteristics of vegetatively propagated crops are important, novelty may command a premium in the ornamental crops. This is in contrast to highly domesticated, seed-propagated crops where a new mutation must be discarded if the new gene(s) cannot be introgressed without a yield penalty (Brock, 1977).

Advances in non-conventional breeding methods (Cassells and Jones, 1995) have arisen from developments in plant tissue culture and molecular biology (genetic engineering), and have been used, in some cases, by researchers who have no formal training in plant breeding or plant pathology. This has led to a failure to appreciate practical problems in mutation breeding for disease resistance

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(Jones and Cassells, 1995). A conventional plant breeding programme has three stages (Simmonds, 1979): (1) the creation of variation, (2) the selection of useful variants and (3) field trials to confirm the performance of the selected variants. No matter how genetic changes are achieved there is the likelihood that finely tuned genomes will be perturbed. Where the crop is seed-propagated and the character is based on one or few genes, back-crossing may be used, but not guaranteed, to reduce associated pleiotropic effects. Equally problematic is the selection of potentially useful variants. Some plant breeders using 'non-conventional' approaches have attempted to achieve one-step plant improvement, regenerating plants adventitiously, for example, in the presence of fungal toxins (Behnke, 1979). Improved cultivars have been claimed on the basis of the selection of plants on media containing uncharacterized extracts of fungal culture media with no supporting trials data (Wenzel and Foroughi-Wehr, 1993). Unfortunately, these publications stand in the literature, and the false expectations they have raised have somewhat tarnished the image of *in-vitro* mutation breeding.

Here, the efficient integration of *in-vitro* mutation breeding and selection into the methods of plant breeding will be considered.

Tissue Culture Systems for Induced Mutagenesis

Tissue culture systems for mutagenesis can be divided into cell and complex explant cultures where plant regeneration is via adventitious buds with or without an intervening callus phase; and bud (meristem or nodal) culture where regeneration is from organized meristems with or without axillary bud proliferation (George, 1993). It is important that in using tissue culture systems only disease-free aseptic cultures are used. (For reviews on the elimination of contaminants from *in-vitro* cultures and for the maintenance of clean cultures see Cassells, 1991, 1992).

Adventitious Regeneration Systems

Adventitious bud formation can occur directly from individual cells of a complex explant or with intervening callus. Callus cells can be grown in suspension culture and plants regenerated from isolated 'single' cells. It is also possible to isolate protoplasts from leaf or other tissue, and regenerate plants. While it is possible to regenerate many cultivars via adventitious buds and the number of amenable species is continually increasing, in some cases (e.g. woody plants), only juvenile tissue can be used, and consequently it is not possible to use explants from elite genotypes. In several crops adventitious regeneration seems genotype-dependent (see George, 1993 for a comprehensive review).

Somaclonal variation is associated with adventitious regeneration and may be a source of background variation in any non-conventional breeding programme that uses this system. Somaclonal variation is genome-dependent, is particularly associated with polysomatic tissues, and influenced by the culture medium and dura-

tion of culture (Karp, 1991). A postulated advantage of adventitious regeneration is that the regenerants are solid mutants (Broertjes and van Harten, 1988, but see Cassells, 1985). Thus, there is a potential for regeneration of non-chimaeral mutants.

Shoot Culture Systems

Meristem culture (for application purposes, here the terms shoot-tip culture, meristem culture, bud-tip culture and nodal culture are regarded as synonymous) is possible for most species. The procedures and media requirements are less stringent for larger bud-tip explants and nodes than those for cells and complex explant culture (for a detailed review of this subject and media formulations see George, 1993).

Meristem and nodal cultures are the preferred systems for cloning plants *in-vitro* as the frequency of spontaneous mutation is low. They represent the conventional targets, i.e. totipotent cells for mutation breeding with the advantage over *in-vitro* vegetative propagules that they are miniaturized and so large numbers can be treated as in many seed-propagated crops. This is an important practical consideration, since about 90% of the commercial mutants have been produced by physical mutagenesis (Micke and Donini, 1993). A perceived disadvantage of irradiating meristems is the creation of unstable chimaeras (Broertjes and van Harten, 1988).

Chimaerism and In-vitro *Culture*

Amongst the recognized problems in exploiting induced mutagenesis with seeds or *in-vivo* vegetative propagules, such as cuttings, is the high incidence of unstable chimaerism resulting from mutated cells in the histogenic layers of the apex giving rise to unstable chimaeras (Cassells and Periappuram, 1993). The problem can be overcome in seed-propagated crops by going through a seed generation; however, in vegetatively propagated crops the breeder must wait for diplontic selection to operate (Balkema, 1972), and successive propagations are necessary to obtain solid mutants. Similar problems of chimaeras in the progeny from adventitious regeneration (Cassells *et al.*, 1986) and irradiation of buds *in vitro* (Sonnino *et al.*, 1986) have been reported.

The Influence of In-vitro *Culture on Plant Quality*

Apart from spontaneous mutations associated with adventitious regeneration, non-heritable changes may also occur in cells and tissues cultured *in vitro* and after establishment in soil. Among *in-vitro* changes that are relevant for selection for disease resistance, are alterations associated with 'vitrification'. These include hyper-hydrated cells with thin walls, hypolignification, poor cuticular development and non-functional (open) stomata (Ziv, 1991). These changes in field-grown plants are generally associated with increased susceptibility to disease

(Isaac, 1992), and may reflect the susceptibility of microplants to damping-off diseases. Changes associated with microplant progeny in the field include altered maturation, which in early and late blight of potato is associated with increased resistance (Russell, 1978), that is, late-maturing mutants express spurious resistance. These maturation mutants are unstable (Cassells *et al.*, 1991) and resemble genotrophs (Nagl, 1990) in being induced, at least in some genotypes, at high frequency. They represent a significant source of unstable, i.e. worthless, variation in potato somaclones (Cassells and Sen, 1995; Kowalski and Cassells, 1995).

Physical and Chemical Mutagenesis

Mutants may be induced *in vitro* by chemical treatment or by physical mutagens (Anonymous, 1977). Physical mutagenesis is considered to be more or less cultivar independent, and this perhaps explains why it is more widely used (Micke and Donini, 1993). That the spectrum of mutants differs between physical and chemical mutagenesis should not be overlooked when deciding which general approach to use (Anonymous, 1977). X-rays induce a different spectrum of mutations to gamma-rays, and various chemical mutagens similarly induce a different spectrum of mutants. Physical mutagens give higher frequency of chromosomal changes; chemical mutagens give predominantly gene mutations. It is unclear whether spontaneous mutation ('somaclonal variation') results in a different pattern of mutagenesis (Karp, 1991).

Physical Mutagenesis

Experimental details for X-ray and gamma-ray mutagenesis are given in Anonymous (1977) and Broertjes and van Harten (1988), and instructional videos are available from the International Atomic Energy Agency, Wagrammerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria. An aspect of physical mutagenesis that should be considered in designing experimental treatments is that radiation sensitivity is strongly influenced by environmental and biological factors. The principal environmental factors are oxygen and tissue water content; radiation damage is inversely related to both factors. Thus tissue cultures differ in their radiation sensitivity from dry seed. Large differences in radiation sensitivity between species have been reported but differences between varieties are usually small (Anonymous, 1977).

Chemical Mutagenesis

While a wide range of chemicals have been reported to be mutagenic, alkylating agents and azides are the most widely used chemical mutagens (Anonymous, 1977). These are potential carcinogens and should be handled with care (see reference to IAEA instructional videos above). Chemical mutagens are reported to

induce a higher rate of gene mutations than physical mutagens but problems in their use, mainly relating to uptake of the chemicals, persistence or residual toxicity, along with general safety aspects have mitigated against their widespread application (Micke and Donini, 1993). Depending on the explant, some of the above difficulties can be minimized by *in-vitro* applications, e.g. by treatment of cells and calli, permeability barriers present in intact tissues are eliminated (see above). In the case of complex explants, chemical penetration problems are also reduced. Where *in-vitro* tissues are vitrified, penetration barriers may also be reduced. These factors should be taken into consideration when designing experiments to optimize chemical mutagen concentration for *in-vitro* use.

Screening for Disease Resistance

Selection in mutagenized populations is complicated by the high probability of multiple mutational events in individual genomes. The issue of chimaerism aside (see above), mutations induce, primarily, recessive alleles and affect gene regulation. While adverse pleiotropic changes may occur in any genome manipulation, from conventional crossing to genetic transformation and in highly domesticated genomes, this problem is increased in induced mutation breeding. There is the need for multi-locational trials for several years in mutation breeding for disease resistance to ensure that the genome–environmental interaction between the crop, the target pathogen and other crop pathogens, reflects sufficient seasonal fluctuations in host susceptibility and pathogen inoculum potential (Niks *et al.*, 1993).

A major consideration in evaluating plant populations is how efficiently screening can be achieved, so that the number of individuals progressing to field trials is reduced, allowing early large-scale trials with the selected progenies that are necessary to conduct meaningful evaluation of agroecological influences on yield, disease resistance, etc. (Romagosa and Fox, 1993). Here, aspects of selection *in vitro*, early selection *in vivo* and mature plant screening will be considered.

In-vitro *Selection*

One facet that distinguishes current mutation breeding from earlier usage, is to exploit the potential of *in-vitro* culture techniques to select improved genotypes. This is evident in studies on somaclonal variation for disease resistance in which many researchers have used some form of *in-vitro* selection. Unfortunately, this has been mostly unsupported by adequate field trials, so the value of selection in many cases remains unconfirmed. Nevertheless, there have been enough studies on genes for disease resistance, followed by field trials, to suggest that this approach may have potential. Selection for resistance against undefined pathogen culture filtrates appears to have resulted in improved disease-resistant lines (e.g. Gengenbach *et al.*, 1977; Chen *et al.*, 1989; Hunold *et al.*, 1992; Toyoda *et al.*, 1991; but see Wenzel and Foroughi-Wehr, 1993). An argument has been made for

the use of host-specific toxins in the selection of resistant mutants (Buiatti and Ingram, 1991) but only a few diseases have host-specific toxins for use in *in-vitro* selection (Jones, 1990), in other cases the situation is unclear e.g. *Alternaria* spp. produce both non-host-specific and host-specific toxins (Rotem, 1994). In other models, the pathogen has been cultured on the medium, then removed or killed, before the culture of plant material. The latter can be cells, callus, complex explants or nodes from mutagenized populations. Validation of selection systems has been based on screening of host genotypes of known resistance status (see Jones, 1990, for a discussion of toxin selection strategies).

In some cases, *in-vitro* toxin-selected mutant lines have been shown to lack vigour in the field (Wenzel and Foroughi-Wehr, 1993) but this is explicable in terms of background mutations, and in studies in which the resistant mutant has been back-crossed to the parental line, stable major gene resistance has been confirmed (Toyoda *et al.*, 1989).

Cassells and Walsh (1995) used an indirect strategy to select for *Sclerotinia* resistance *in vitro*. Having failed to select for resistant somaclones using oxalic acid (a determinant of pathogenicity), they used a reverse selection for the ability of somaclones to grow on low calcium (a determinant of host resistance to *Sclerotinia*; calcium in the host counteracts the action of oxalic acid produced by the pathogen). Resultant somaclones were resistant to mechanical inoculation with the pathogen and showed improved field resistance. *In-vitro* selection for enhanced expression of determinants of host resistance, e.g. antibiotic compounds (Isaac, 1992), merits further investigation.

It is recognized that hypersensitivity expression requires intercellular communication, and consequently, this valuable form of resistance cannot be selected in disorganized cell and callus cultures (Wenzel and Foroughi-Wehr, 1993).

Co-cultivation of Pathogen and Induced Mutations

There have been several approaches to co-cultivation of pathogen and putative mutant (usually somaclonal) populations (Meulemans *et al.*, 1987; Dunbar and Stephens, 1989). They include inoculation of the plant tissue culture with pathogen inoculum, inoculation of the plant tissue with obligate pathogens or co-cultivation on compartmentalized vessels where pathogen metabolites can diffuse towards the plant tissues. Technically, these systems can be difficult to establish, e.g. there is the problem of eliminating bacteria from fungal inoculum, of maintaining pathogen virulence, etc. A further complication is that microplants (like seedlings), and particularly vitrified tissues, cells and calli, do not express many of the constitutive resistance factors formed in mature plants. Microplants, especially vitrified tissues, are not lignified and so poorly represent mature plant resistance (Ziv, 1991). Correlation between resistance to pathogen inoculation *in vitro* and in the field has been poor (Meulemans *et al.*, 1987; Cerato *et al.*, 1993).

Screening of Induced Mutants as Young Plants In Vivo

Race non-specific resistance may not be expressed in young plants. A further complication in screening microplant progeny is prolonged juvenility associated with plants produced *in vitro* (George, 1993), e.g. resistance to foliar blight in potato is related to leaf maturity (Cassells *et al.*, 1991). Juvenile foliage is more resistant; in tubers the situation is reversed, older tubers being more resistant (Umaerus and Umaerus, 1994).

Screening of Mature Plants

Screening of plants raised in protected environments, ranging from phytotrons to glass or screen-houses, and controlled inoculation, to study specific host–pathogen interactions, can be a valuable pre-screening approach to discard worthless lines for field evaluation. For plants normally grown as protected crops, these methods can give definitive results provided that there is adequate environmental control and representative pathogen inoculum. In many cases, detached (mature) leaves, in Petri dishes, have been used for large-scale intensification of screening (Umaerus and Umaerus, 1994; Niks *et al.*, 1993). Again, in some pathogen–host combinations, a good correlation has been shown with whole plant resistance; in other models, leaf maturity has been a problem (Russell, 1978). In all 'artificial' screening, the issue is how closely the conditions represent natural infection pressure. The system should be established to represent the natural pathogen–host–environment interaction (use of appropriate inoculum; type, e.g. sexual/asexual spores, amount; tissue, temperature, etc.) to ensure that disease escape is not confused with disease resistance. A deficiency of artificial screening is that character defects in the lines may not be expressed. This is of importance in relation to mutation breeding and the occurrence of recessive mutations; that is, decreased resistance to non-selected pathogens may emerge. Therefore, trials, carried out on field crops under protected cropping or artificial inoculation, cannot confirm retention of desirable traits, including yield.

Field Trials for Disease Resistance

A crop can be exposed to a range of soil and cultural conditions over its geographical distribution which exert a strong influence on gene expression (Romagosa and Fox, 1993). Similarly, the inocula of pathogens affecting a crop are strongly influenced by the environment (Scott and Bainbridge, 1978). It has long been recognized by conventional plant breeders that multi-location trials in successive seasons are required to assess the stability of resistance. The elements of conventional selection and evaluation involve screening of large mutagenized populations under glass or in the field; this is followed by exposure of the selected lines to high selection pressure of the pathogen, followed by multi-locational trials in the cultivar growing area (Russell, 1978; Niks *et al.*, 1993). For late blight

resistance screening, Umaerus and Umaerus (1994) recommended growing in Mexico where both pathogen (*Phytophthora infestans*) variability and inoculum pressure are high.

Strategy for *In-vitro* Breeding for Disease Resistance

Several conventional and non-conventional methods are now available to the plant breeder (Cassells and Jones, 1995). In deciding which is the most appropriate and cost-effective strategy in disease resistance breeding, the main factors that must be considered are the breeding system of the crop, the type of resistance sought and the extent of background change which can be tolerated. In induced versus exploitation of spontaneous *in-vitro* (somaclonal variation) mutation in plant breeding, in addition to a tissue culture laboratory, facilities for physical or chemical mutagenesis must also be available (Jones and Cassells, 1995). Here, a comparison is made between mutation breeding and conventional cross-breeding. The question of when to use mutation breeding has been discussed by Brock (1977) and Micke and Donini (1993).

Crops can be divided into four main groups: in-breeding species, out-breeding species, vegetatively propagated species capable of sexual reproduction, and sterile species. Resistance can be divided into two categories: major gene (race specific) and minor gene (quantitative, non-race specific).

Induced major gene resistance, involving one or a few genes, can be exploited efficiently in seed-propagated species where back-crossing eliminates background damage in induced mutants (Brock, 1977). Mutation breeding has been used frequently and with success in crop improvement (Maluszynski *et al.*, 1995). Nevertheless, conventional breeding for disease resistance is preferred rather than mutation breeding where resistance genes are available in the interbreeding population. Similar considerations apply to minor gene manipulations in the in-breeding species where background-induced mutations in highly domesticated crops are likely to reduce yield.

Mutation breeding in diploid out-breeding species is more problematic than in the in-breeding species, because of the difficulties in selecting recessive mutations. As with fertile species where resistance exists in cultivars or related species, sexual crossing may be the preferred breeding strategy. Induced selfing (e.g. by bud pollination) or sib-mating may be necessary if mutation is to be used. With minor gene characters, mutation breeding is not recommended because of yield depression, unless no natural variability exists.

In out-breeding polyploids, e.g. potato (Bradshaw and Mackay, 1994), where massive segregation occurs in sexual reproduction breaking up desirable gene combinations, it can be difficult to recombine resistance with existing varietal characters. Here, marker-assisted selection has an important role (Arus and Moreno-Gonzalez, 1993). Historically, spontaneous mutations have been exploited. It follows that for this group, and for sterile species, mutation breeding for disease resistance has application as demonstrated by the number of mutants

registered for horticultural crops (Broertjes and van Harten, 1988; Micke and Donini, 1993). It is in crops with these breeding systems, that spontaneous and induced mutation *in vitro* has the greatest application, particularly when high yield may not be essential for commercial exploitation. Nevertheless, many reports show that induced mutagenesis has an established role in the improvement of disease resistance in many seed-propagated species (Maluzynski *et al.*, 1995).

The Future for *In-vitro* Mutation Breeding

Developments in molecular biology have provided plant breeders with new tools for plant improvement. Both genetic engineering and marker-assisted selection have major contributions to make (Cassells and Jones, 1995). However, unwanted somaclonal variation in some tissue culture systems, background changes due to multiple gene insertions resulting in negative sequence interactions and insertion positional effects are major problems of transgenic breeding (Meyer, 1995). As in mutation breeding, introgression to eliminate these effects may be required but limited by the factors discussed above. Another consideration is the move from emphasis on breeding from major to minor gene resistance. While transgene 'stacking' is a possible option, the limitations to introgress transgenes must be recognized. Further, the search for suitable resistance genes is only beginning, and the question of whether the transfer of resistance genes across species boundaries will be matched by the transfer of virulence factors between species, particularly bacterial species, remains. It is reasonable to postulate that mutation breeding will continue where it has established a niche, e.g. in breeding for disease resistance in seed-propagated species as historically used and in out-breeding polyploid and seed-sterile cultivars. The efficiency of mutation breeding has been increased by the availability of *in-vitro* propagules, and will also benefit from the development of early pre-screening systems.

Mutagenesis of buds is associated with chimaerism. The positive aspect of chimaerism, namely diplontic selection, i.e., the selection of 'fit' mutant cells, has been overlooked (Cassells *et al.*, 1993). Irradiation of apices followed by chimaeral dissolution may be the most efficient use of *in-vitro* mutation breeding in crops where the desired mutant character must be combined with retention of vigour, that is, where vigour (as expressed by competitiveness at the cell level in diplontic selection) is correlated with yield. Once chimaeras have been broken down by at least three cycles of subculture *in vitro* (Sonnino *et al.*, 1986; Cassells *et al.*, 1993), the putative homohistonts may be subjected to *in-vitro* selection. Empirical selection against fungal metabolites appears to result in potentially useful variants. But there are other options, e.g. selection could be made for overproduction of constitutive defence factors, e.g. increased cuticular waxes, increased wall lignification or methylation, increased calcium accumulation, increased phytoalexin production, etc. (Isaac, 1992). Mutation breeding has been used to suppress many host resistance genes, e.g. for spine production, bitter taste, texture, etc., in the domestication of plants. The reversal of some of these

mutations by de-repression of synthesis and multiple mutations or amplifications in constitutive minor genes conferring resistance may lead to more durable disease resistance. In the short to medium term, empirical mutation breeding may offer the best option to produce durable resistance in vegetatively propagated crops or where natural resistance genes are lacking. The efficiency of the induced mutation can be increased by screening against unwanted genotypes and selection for improved genotypes (Cassells and Sen, 1995; Kowalski and Cassells, 1995).

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19. EMS and Transposon Mutagenesis for the Isolation of Apomictic Mutants in Plants

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Introduction

Apomixis is an asexual mode of reproduction through seed. It is widespread throughout the plant kingdom. Among angiosperms, more than 300 plant species from 35 different families have been described as apomictic. Apomixis is most common in Gramineae, Compositae and Rosaceae (Rutishauser, 1967; Nygren, 1967; Bashaw, 1980; Nogler, 1984; Hanna and Bashaw, 1987; Asker and Jerling, 1992; Hanna, 1991, 1995a,b; Koltunow, 1993; Khush *et al.*, 1994). Apomixis is strongly correlated to polyploidy and hybridization, and it is more prevalent in the polyploid wild species. It is also widespread in several forage grasses, such as *Cenchrus, Eragrostis, Paspalum, Poa* and others, most of which are polyploid.

Apomixis offers various benefits for crop improvement:

1. Apomixis allows vegetative reproduction through the seed, and thus can have a major impact not only in seed-propagated crops (cereals, vegetables), but also in vegetatively-propagated crops (e.g. potato).
2. A major advantage of apomixis is in F_1 hybrid development for commercial seed production. It simplifies seed production, because isolation is not necessary, and there is no need to maintain or multiply parental lines. As an alternative to current commercial hybrid production systems, it would make hybrids readily available and affordable, reducing the costs of seed production. Thus, hybrid vigour can be exploited advantageously in vegetables, wheat, soyabean, rice, minor grains, forage and turf species, if apomixis genes could be introduced into the target crops.
3. Apomixis makes it possible to fix the genotype of a superior variety bred for a particular environment, so that clonal seeds can be produced continuously and economically.
4. Progeny testing for genetic stability is not needed for obligate apomixis, since any superior apomictic lines are ready for immediate performance evaluation.

Although apomixis was described as early as 1841, and the genetics has been investigated for several years, progress has been limited in its use for crop improvement (Asker and Jerling, 1992; Hanna, 1995a). One of the main reasons is that apomixis is usually found in distantly related polyploid wild species. The transfer of apomixis by classical methods of interspecific hybridization is difficult and time-consuming due to sexual incongruity, and several other problems, such as

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lack of introgression, occurrence of male sterility, poor seed-set and low frequency of apomictic progeny plants. Further, the obligate apomict can only be used as a male, and the facultative apomict as a female. In the case of hybridization with diploids, usually the best results are obtained when the polyploid is used as a female.

With recent progress made in cellular and molecular biology, it should be possible to isolate the genes controlling apomixis and to introduce them through transformation into commercially important crops. Currently, several laboratories are engaged in the isolation and transfer of genes for apomixis, for example in *Pennisetum*, maize and *Brachiaria* (Ozias-Akins *et al.*, 1993; Hanna, 1995a,b; Savidan *et al.*, 1993; Liu *et al.*, 1994; Kindiger *et al.*, 1996). Mapping populations have been developed in *Pennisetum* and *Tripsacum*–maize hybrids. In addition, mutational approaches are being followed to induce mutants for apomixis and isolate genes in *Arabidopsis*, and rice (Chaudhury and Peacock, 1993; Khush *et al.*, 1994; Peacock *et al.*, 1995; Koltunow *et al.*, 1995; Ohad *et al.*, 1995). Recently, in *Arabidopsis*, EMS-induced mutants for fertilization-independent seed development (*fis*) and fertilization-independent endosperm (*fie*) mutants, associated with apomixis, have been reported (Peacock *et al.*, 1995; Ohad *et al.*, 1995). In this chapter, we describe the strategies of transposon tagging and EMS mutagenesis for the induction of apomictic mutants in *Arabidopsis* and *Petunia*, which enable the isolation of genes controlling apomixis. The rationale of various genetic screens and the progresss made will be discussed.

Sexual versus Apomictic Reproduction

The induction of mutants and isolation of genes controlling apomixis require a thorough understanding of various types or mechanisms and the genetics of apomixis. In angiosperms, a sequence of events occurs to form seed through sexual reproduction: (a) megasporogenesis, (b) megaspore selection, (c) megagametogenesis, and (d) double fertilization.

(a) *Megasporogenesis*: megaspore mother cell differentiates from a cell near the micropyle in the hypodermal layer of the nucellus. The megaspore mother cell undergoes meiosis-I to form two spores, followed by a mitotic-like division (meiosis-II), and results in a tetrad of megaspores, each with a haploid-set of chromosomes.
(b) *Megaspore selection*: from the tetrad of megaspores, the three megaspores nearest the micropyle degenerate, while the functional chalazal megaspore enlarges to form an embryo sac located at the micropylar end of the ovule.
(c) *Megagametogenesis and embryo sac maturation*: the nucleus undergoes three mitotic divisions usually forming an octo-haploid nucleate embryo sac with an egg, two synergids, two polar nuclei and three antipodals.
(d) *Double fertilization*: one sperm nucleus unites with the egg cell to form a diploid zygote, which gives rise to the embryo. The other sperm nucleus

unites with the two polar nuclei, which fuse to a central nucleus. The resulting triploid cell gives rise to the endosperm. The synergids degenerate at the mature embryo sac stage, and the antipodals may continue to divide and form a cluster of cells at the chalazal end of the embryo sac opposite the egg. Great variation exists for antipodal proliferation among genotypes, species or genera (Hanna, 1991).

Mechanisms of Apomixis

In apomixis, a cell with the unreduced (somatic) chromosome number or restitution originating from either somatic cells or germinal cells in the ovule develops into an embryo without the union of egg and sperm nuclei. Apomictic processes deviate from sexual reproduction in several aspects: (i) modification or complete avoidance of meiosis, (ii) formation of unreduced megaspores, (iii) parthenogenetic development of embryo, and (iv) autonomous or pseudogamous endosperm development without fertilization (Rutishauser, 1967; Asker, 1980; Nogler, 1984; Asker and Jerling, 1992; Koltunow, 1993). Apomixis is broadly classified into two types, i.e. gametophytic apomixis and adventitious embryony.

Gametophytic Apomixis

This type of apomixis is initiated early in ovule development as compared to adventitious embryony. Gametophytic apomixis results in the formation of a megagametophytic structure without meiotic reduction, and the embryo develops from a cell inside this unreduced megagametophyte. Gametophytic apomixis can be obligatory, where sexual reproduction is essentially excluded, and all the seed progeny have the genotype of the female parent. It can also be facultative, where both apomictic and sexual forms occur, i.e. sexual and apomictic processes can coexist within the same ovule of the plant. Thus, both zygotic and apomictic seeds can be obtained. Gametophytic apomixis is strongly correlated to polyploidy, and is rare in diploids. Gametophytic apomixis is divided into two categories: diplospory and apospory. In both of these, an embryo sac is formed, and the two types are distinguished by the origin of the cells that give rise to the apomictic embryo sac.

Diplospory

Diplospory has been reported in several species, belonging to genera, such as *Eragrostis, Tripsacum, Elymus, Taraxacum, Ixerix, Parthenium, Potentilla* and several other species (Brown and Emery, 1958; Bashaw, 1980; Nogler, 1984; Hanna, 1991; Koltunow, 1993). A megaspore mother cell differentiates from a hypodermal cell of the nucellus as in sexual reproduction, but afterwards the cell development proceeds in different ways. The embryo develops parthenogenetically from the unreduced egg, and the endosperm develops autonomously from the

unreduced polar nuclei without fertilization, e.g. Compositae. The mechanisms which initiate these events are not known. Autonomously produced endosperm can have variable ploidy levels, suggesting that the fusion of the polar nuclei is not a prerequisite for the initiation of mitotic activity in the endosperm (Nogler, 1984; Bashaw and Hanna, 1990; Asker and Jerling, 1992). In a few species (e.g. *Eragrostis, Tripsacum*), pollination is necessary for endosperm production, i.e. the selective fusion of an unreduced polar nucleus with a sperm nucleus (pseudogamy). The mechanism that prevents the fertilization of the unreduced egg cell is not understood. In *Tripsacum*, embryo development begins before anthesis (Asker and Jerling, 1992). The absence of meiotic reduction or lack of linear tetrad of megaspores is the best evidence of diplospory (Bashaw, 1980). There are three categories of diplospory which are based on the cytological events, i.e. meiotic diplospory, mitotic diplospory and *Allium*-type of diplospory (Nogler, 1984; Koltunow, 1993).

(a) *Meiotic diplospory*: this occurs in *Taraxacum, Arabis, Paspalum* and some genera of compositae. A megaspore mother cell differentiates from the nucellus and enters meiotic prophase, but chromosomes do not pair due to asynapsis. After the first meiotic division, a restitution nucleus forms which eventually divides mitotically to form a dyad with somatic (2n) chromosome number, and eventually it results in an eight-nucleate embryo sac. In *Ixerix*, the meiotic process is slightly different from the above. The megaspore mother cell undergoes asyndetic meiotic prophase, eventually resulting in a restitution nucleus. Afterwards, a division similar to meiosis-II takes place without cytokinesis. An embryo sac with eight nuclei forms after two mitotic divisions of the unreduced nuclei.

(b) *Mitotic diplospory*: this is also called the *Antennaria* type of apomixis, which is the most common form of diplospory with a wider taxonomic distribution (Nogler, 1984). This type of diplospory is characterized by a complete avoidance of meiosis, where megaspore mother cell functions as an unreduced megaspore. Afterwards, it undergoes mitotic divisions resulting in the formation of a typical eight-nucleate unreduced embryo sac.

(c) *Allium type of diplospory*: in this type, premeiotic chromosome doubling occurs through endoreduplication or endomitosis, resulting in the formation of two identical chromosomes aligned in parallel close together, both with two chromatids. This is followed by meiosis, where pairing between sister chromosomes takes place forming auto bivalents, and thus no gene segregation occurs because the chromosomes involved in the bivalents are identical (see Sybenga, 1992). This results in a tetrad of unreduced nuclei of parental genotype. Two subsequent mitoses in the chalazal dyad result in eight-nucleate embryo sac. The polyploid species, *Allium nutans* (2n = 40), *A. odorum* (2n = 32) and *A. tuberosum* (2n = 32) show this type of diplospory (Håkanson and Levan, 1957; Gohil and Kaul, 1981; Kojima and Nagato, 1992). Indian *A. tuberosum* (Chinese chives), which is commercially important, also has sexual forms.

Apospory

In apospory, unreduced embryo sacs arise from somatic cells of the nucellus. Apospory is the most common apomictic mechanism in a large number of grass species belonging to *Pennisetum, Poa, Paspalum* and *Cenchrus* (Nogler, 1984; Hanna and Bashaw, 1987; Hanna, 1995a,b). Aposporous and sexual processes can coexist within a given ovule. At the megaspore mother cell stage, the sexual processes are often terminated in many apomictic species (Nogler, 1984; Asker and Jerling, 1992). If the aposporous initials differentiate late when the formation of the sexual megametophyte is relatively advanced, both types of embryo sac may exist. The formation of the number of embryo sacs can depend on the species (Nogler, 1984; Bashaw and Hanna, 1990). Aposporous embryo sacs can be distinguished from the sexual embryo sacs by the lack of antipodals (as in *Panicum* type), size, shape and location of the embryo sac, and the number of nuclei and their location in the embryo sac (Hanna, 1991). In *Hieraceum*, an unreduced eight-nucleate bipolar embryo sac is formed, whereas in *Panicum* an unreduced four-nucleate monopolar embryo sac is the rule, though in certain cases embryos are bipolar eight-nucleate. A single cell of the embryo sac develops into an embryo parthenogenetically. Whether this occurs by the same mechanism as that in diplospory is not known. For endosperm development, pollination is necessary in most species, except in *Hieraceum* (Asker, 1980; Nogler, 1984; Bashaw and Hanna, 1990; Asker and Jerling, 1992).

Adventitious Embryony

In adventitious embryony, embryos arise directly from the individual cells of the somatic tissues, i.e. nucellus or integuments external to a sexual embryo sac in the mature ovule, and is purely a sporophytic form of apomixis (Bashaw, 1980; Lakshmanan and Ambegaokar, 1984). Adventitious embryony occurs in relatively fewer plant species than diplospory or apospory. Adventitious embryony has been an important mode of reproduction in *Citrus* for propagating and maintaining virus- and disease-free rootstocks (Parlevliet and Cameron, 1959). In *Citrus*, sexual and apomictic processes occur concurrently within the same ovule, and the apomictic embryo development from the nucellus is morphologically similar, if not identical, to the stages of sexually derived embryo sac development. It is coupled to pseudogamy, and is therefore dependent on the presence of a sexual embryo sac in the same ovule to develop an endosperm for the nourishment of the apomictic embryo (Koltunow, 1993). Thus, it occurs in the presence of normal sexual reproduction, and results in polyembryony.

Identification of Apomixis

Several screening techniques can be used to identify various apomictic mechanisms, based on cytological, genetic and biochemical/molecular observations.

These include: cytology of the embryo sac development, the pistil-clearing technique (Young *et al.*, 1979; Crane and Carman, 1987), callose fluorescence combined with pistil-clearing, histological serial sections of ovules, isozyme and RFLP markers (Matzke, 1991; Mazzucato *et al.*, 1995; Naumova, 1993; Ozias-Akins *et al.*, 1993; Wagenvoort *et al.*, 1995) and progeny tests (Hanna, 1991, 1995a; Khush *et al.*, 1994). Cytological observations are more rapid than progeny tests. The pistil – or ovule-clearing techniques (Young *et al.*, 1979; Crane and Carman, 1987) are widely used for examination of the embryo sacs. Apospory can be identified by the presence of multiple embryo sacs, lack of antipodal development, and/or shape and orientation of embryo sacs in the ovule. Callose fluorescence is used in combination with pistil clearing to detect diplosporous embryo sac development. Carman and Wang (1992) reported that the lack of fluorescing callose in the walls of dyads, tetrads and megaspore mother cells is an indication for diplospory. Also molecular markers, such as RFLP to the apomixis gene(s) can be used to identify the apomictic plants in the seedling stage. Hanna *et al.* (1993) and Ozias-Akins *et al.* (1993) used this method to screen for apomixis in BC progenies derived from crosses of two wild species of *Pennisetum* with sexual pearl millet.

Genetics of Apomixis

Because of the polyploid nature of many apomictic species and the complex nature of the apomictic mode of reproduction (Asker, 1980; Hanna, 1991, 1995a,b), the results of crosses between apomictic and sexual plants have not been conclusive. Several studies have shown that the ability to reproduce apomictically is genetically determined. Apomixis appears to be controlled by one or a few genes that are dominant in some species, and recessive in others (Asker, 1980; Bashaw, 1980; Nogler, 1984; Hanna, 1995a). The occurrence of obligate and facultative apomixis and varying degrees of facultative apomixis in the same species indicates that apomixis may be affected by modifier genes and/or genetic background. Taliafero and Bashaw (1966) reported that two genes exhibited epistatic control of apospory in *Cenchrus ciliaris.* Burton and Forbes (1960) obtained similar results which suggest that apomixis in *Paspalum notatum* is recessive to sexuality and controlled by a few genes. Sax (1959) reported that apomixis in apple is dominant over sexual reproduction. Funk and Han (1967) suggested that apomixis in *Poa pratensis* may be controlled by two or more dominant genes. Savidan (1980, 1981) reported that apospory is controlled by a dominant gene in *Panicum maximum.* Asker (1970) found apospory in *Potentilla* to be recessive. Previous studies in *Citrus* suggest that adventitious embryony (nucellar embryony) is controlled by a single dominant locus (Parlevliet and Cameron, 1959). The investigations in *Pennisetum* and *Ranunculatus* suggest that a single dominant locus controls apospory in these species (Dujardin and Hanna, 1983; Nogler, 1984; Asker and Jerling, 1992). Apospory in the wild species *Pennisetum squamulatum* has been shown to be associated to a single chromosome segment (Dujardin and Hanna, 1989). Molecular markers linked to the apomixis trait have

been described (Ozias-Akins *et al.*, 1993; Leblanc *et al.*, 1995). Also, studies in *Taraxacum* suggest that diplospory might be under the control of a single locus (Mogie, 1988).

Transfer of Apomixis from Apomictic to Sexual Species

Interspecific Hybridization

The transfer of genes determining apomixis from apomictic to amphimictic (sexual) species can be achieved by interspecific hybridization between the amphimictic crops and the related wild apomictic species. The prospects of this approach depend on the availability of apomictic wild relatives, the suitable genetic basis of apomixis and the sexual compatibility. Bashaw (1980) developed three improved cultivars of *Cenchrus* through hybridization between sexual and apomictic clones. In *Pennisetum*, the trispecific hybrid obtained between the cultivated sexual species, *P. glaucum* (induced tetraploid, 2n = 4x = 28), the wild apomictic species, *P. squamulatum* (2n = 6x = 54) and a bridging species, *P. purpureum* (2n = 4x = 28), was backcrossed repeatedly to the tetraploid, *P. glaucum*, and eventually an obligate apomictic individual with 29 chromosomes (a monosomic addition plant) was selected (Dujardin and Hanna, 1984, 1989). The *P. squamulatum* chromosome carrying apomixis was thus identified. The apomictic trait has been tagged with closely linked molecular markers (Ozias-Akins *et al.*, 1993). Also attempts have been made to transfer apomixis from the wild species, *Tripsacum dactyloides* (2n = 72) to *Zea mays* (2n = 20). The F_1 hybrid (2n = 46), obtained from the crosses of the two species, has been backcrossed repeatedly to maize, and apomictic BC progenies with 20 maize chromosomes and two to six chromosomes of *Tripsacum* have been selected. Further attempts for the transfer of apomixis are under way (Savidan *et al.*, 1995; Kindiger *et al.*, 1996). In addition, efforts are also being made in *Beta, Malus, Agropyron, Elymus* and *Solanum* for transfer of apomixis (reviewed in den Nijs and van Dijk, 1993; Khush *et al.*, 1994; Hanna, 1995a,b). However, the progress of efforts in various plant species, including *Pennisetum, Tripsacum* and *Cenchrus* to transfer genes for apomixis has been slow and hindered by the higher ploidy levels of the apomictic species, restricted recombination between the chromosomes of wild and cultivated species, and the occurrence of sterility in the progeny plants. Other approaches, such as microprotoplast-mediated chromosome transfer, which enables a direct production of monosomic addition plants between sexually incongruent species (Ramulu *et al.*, 1996a,b), can be a suitable alternative for the transfer of apomixis from the wild to cultivated species.

Mutations for Elements of Apomixis

Another approach, i.e. 'synthesis', involves the combination of different mutations for elements of apomixis, e.g. modification/avoidance of meiosis,

suppression of recombination (meiotic mutants), unreduced egg cell development, parthenogenetic development of embryo and autonomous/pseudogamous endosperm development. Meiotic mutants which are common in higher plants (e.g. '*mei*' mutants in maize, asynaptic or desynaptic mutants in *Pisum*, potato or tomato), could be used to synthesize apomixis (reviewed in Asker, 1980 and Sybenga, 1992). Mutations can affect the synaptic processes and chromosome pairing, and thus influence recombination (Sybenga, 1975). To introduce apomixis into potato, mutants that reduce recombination and stimulate FDR are required (Ramanna, 1983; Hermsen *et al.*, 1985). Several mutants for desynapsis have been isolated in potato (Jongedijk, 1986; Jongedijk and Ramanna, 1988 and Jongedijk *et al.*, 1991). The desynaptic mutants had a very low frequency of bivalents, strongly reduced recombination, and complete maintenance of heterozygosity. Desynapsis stimulates FDR, especially in genotypes with a strong tendency to early centromere activation. By combining proper desynapsis or asynapsis genes and specific genotypes with parthenogenetic (diplospory) ability of producing seeds, it appears to be possible to produce apomictic seed production in potato.

Induction of Mutations for Apomixis

The development of haploid eggs after meiosis and the formation of sexual embryos after fertilization, follow a defined sequence of events: (a) megaspore mother cell differentiation, (b) megasporogenesis through meiosis leading to the development of a haploid megaspore, and (c) gametogenesis, resulting in embryo sac with egg, polar nuclei and antipodal cells. The switch from these sexual processes to an apomictic pathway seems to occur in simple steps. For example, in meiotic diplospory, meiosis is modified resulting in an unreduced megaspore. In mitotic diplospory, the megaspore mother cell directly undergoes mitosis without meiosis. In both cases, embryo sacs with 2n eggs are produced, and the eggs are prevented from fertilization, eventually developing into embryos with the maternal chromosome composition. In apospory, nucellar cells adjacent to the megaspore mother cell differentiate, and give rise to unreduced embryo sacs by a mitotic process. The aposporous embryo sacs develop faster than sexual embryo sacs, because they do not have to go through meiosis. The development of the sexual embryo sac is terminated, and the aposporous embryo sac takes its place at the chalazal end. The unreduced egg develops into an embryo without fertilization. Thus, apomictic reproduction is believed to be caused by the premature activation of embryo sac development, catalysing a cascade of gene action in one or more nucellar cells or an unreduced megaspore resulting in embryo sac formation that bypasses meiosis (see Peacock, 1992 and Koltunow, 1993). This, and the reports mentioned earlier on the involvement of a single locus for apomixis, suggest that it might be possible to mutate a sexual plant into an apomictic one by the mutation of single genes. In this regard, the mutation could remove a putative repressor of apomictic pathway, or it could lead to the induction of a protein capable of initiating the cascade of gene action, leading to apomictic seed devel-

opment (Peacock *et al.*, 1995; Koltunow *et al.*, 1995). The fact that apomixis occurs very widely suggests that such mutations could have occurred in nature. A dominant silencing DNA region could produce a similar effect as that of a single dominant gene resulting in apomixis. The appearance of sexual plants in predominantly obligate apomictic species indicates that reverse mutations at the apomictic locus or a modifier or controlling gene can occur (Bashaw, 1962; reviewed in Khush *et al.*, 1994). Incomplete penetrance of a silencing effect of the DNA region could explain these 'revertants'.

Ionizing radiations and various chemical mutagens are known to induce mutations for different traits and plant processes. However, studies on the induction of mutations for apomixis have been very limited until now. Hanna *et al.* (1970) selected apomictic plants from irradiated progenies of *Sorghum*, and Hanna and Powell (1973) and Arthur *et al.* (1993) obtained facultative apomicts of *P. glaucum*. To mutagenize a sexually reproducing plant and to detect the apomictic mutants is not easy. This is due to the fact that the sexual reproductive system can mask the formation of apomictic progeny, making it difficult to determine whether a sexual or an apomictic pathway was followed during seed development. Therefore, for mutagenic treatment, it is desirable to use male sterile lines for inducing apomictic mutations because, in this case, seed formation will be indicative for the non-sexual mode of seed production. Due to the availability of male sterile mutants and other suitable genotypes in *Arabidopsis*, rice and *Petunia*, efforts are under way for inducing apomictic mutants (Chaudhury and Peacock, 1993; Khush *et al.*, 1994; Peacock *et al.*, 1995; Ohad *et al.*, 1995, and the present study).

EMS Mutagenesis for the Induction of Apomictic Mutations in Arabidopsis

Induction of mutations can be carried out by a variety of techniques. An efficient method to induce mutations is by treatments with ionizing radiations or chemical mutagens. In *A. thaliana*, ecotype cv. Landsberg erecta, previous investigations have shown that ionizing radiations, such as X-rays and fast neutrons, induce chromosome rearrangements, such as reciprocal translocations (Ramulu and Sybenga, 1985), and also point mutations (Koornneef *et al.*, 1982; Ramulu and van der Veen, 1987), while EMS induces mainly point mutations (reviewed in Koornneef *et al.*, 1982). The subsequent sequence analysis revealed that EMS-induced mutations were single base-pair changes, and the radiation-induced mutations were due to large deletions (M. Koornneef, personal communication). Chemical mutagens induce small alterations (point mutations up to 50 bp deletions), whereas ionizing radiations produce larger deletions (15 bp to tens of centimorgans), and gross chromosomal changes, as revealed from studies on *Arabidopsis*, maize and tomato (reviewed in Maluszynski *et al.*, 1995). Most mutations in *Arabidopsis* have been generated using EMS, although many other mutagens are known to be effective. Chemical mutagens can provide the means whereby saturated mutagenesis can be employed to identify the function of genes in a developmental or physiological process. In this regard, a proper design of the

screen is important to detect mutations. Mutagenesis, followed by appropriate biochemical screens, has been very successful in identifying alterations in the ethylene, fatty acid and many other biosynthetic pathways in *Arabidopsis*.

Advantages of *Arabidopsis*

The advantage of *Arabidopsis* lies in its suitability for mutagenesis, molecular genetics and the existence of an international network of scientists using *Arabidopsis* as a model plant for investigations of many aspects of plant growth and development. The small genome size, and the limited amount of repeated DNA, combined with well-established molecular genetic methods, are ideal for the ready mapping and isolation of genes involved in plant processes and development (Meyerowitz, 1989; Koornneef, 1994; Somerville, 1995). *Arabidopsis* is a self-fertilizing hermaphrodite in which plant anthesis and pollinations are completed before the flowers are fully opened. After fertilization, seeds are formed and siliques elongate about five-fold to form full-length seed pods. In the absence of seed formation, e.g. in a male sterile plant, the siliques remain short.

Arabidopsis thaliana is also a very convenient plant species for mutation research. It is of small size, and has a short life-cycle so that a large number of plants can be screened following mutagenesis. Further, the mutants isolated in *Arabidopsis* for ovule-specific genes will not only greatly aid in understanding the genetic regulation of various developmental processes in female gametogenesis, but also the isolated genes can be used as molecular probes for genetic manipulation of apomixis. Also, appropriate mutants can be used to screen for apomictic mutations: e.g. *APETELA3* (*AP3*), *AINTEGUMENTA (ANT*), female sterile mutants, such as short integument (*sin*) and bell-shaped (*bell*), and ovule (*ovm*) mutants (Gasser and Robinson-Beers, 1993; Reiser and Fischer, 1993; Elliott *et al.*, 1996). In *AP3* and *pistillata* (*pi*), the third whorl of stamens is missing (Coen and Meyerowitz, 1989), whereas some *eceriferum (cer)* mutants show conditional male sterility at low relative humidity. Recently, *pistillata* and *cer* mutants have been used for the induction of apomictic mutations after EMS treatments (Peacock *et al.*, 1995; Ohad *et al.*, 1995). For *pistillata*, both recessive and dominant screens have been devised, and *fis* mutants were isolated (Peacock *et al.*, 1995; Koltunow *et al.*, 1995). Also, in the case of the *cer* line, *fie* mutants have been isolated (Ohad *et al.*, 1995).

Conditional Male Sterile '*cer*' System

To screen for apomictic mutations after EMS treatment, we have used the conditional male sterile *eceriferum* mutants (waxless), *cer1* and *cer6–2 (pop1)* (Koornneef *et al.*, 1989; Preuss *et al.*, 1993). When the *cer* plants are grown at low humidity (about 50% RH), the pollen which is formed does not germinate, and thus no self-fertilized seeds can be obtained (siliques remain short). On the other hand, when the plants are subjected to high humidity (90% RH) at flowering, pollen germinates and normal fertilization takes place, resulting in long

siliques with seeds. In the *cer* mutants, pollen is deficient in long-chain lipids, and contains no lipoidic tryphine, which is necessary for pollen germination, and thus for fertilization. The lipids, such as alkanes, secondary alcohols and ketones, reside within the tryphine that coats the surface of pollen grains. At low humidity, pollen does not absorb water from the stigma (no hydration), and therefore no germination of pollen occurs. The stigma cells, that contact the pollen, produce callose. Thus, at low humidity, tryphine is required for pollen germination and for mediation of pollen–stigma signalling. High humidity results in pollen hydration, and thus in pollen germination and fertilization.

EMS Treatment

The concentrations of EMS (45, 60 and 75 mM) were chosen on the basis of our previous experiments (Ramulu and van der Veen, 1987). After a 5 h treatment, the seeds of *cer1* or *cer6–2* were repeatedly washed with water to remove the remaining EMS and other hydrolytic products that cause toxic effects. The ovules of the M_1 plants were screened for ovule fertility and the M_2 embryos (formed from fertilized ovules) for mutants, such as embryonic lethals and chlorophylla (*albina, xantha, viridis*) to analyse the mutagenicity of EMS treatments. The given concentrations were highly effective in inducing a high frequency of M_2 mutations (embryonic lethals and chlorophylla).

Genetic Screen for the Detection of Recessive Apomictic Mutations after EMS Treatment

To detect recessive apomictic mutations, several thousand *cer1* and *cer6–2* seeds were treated with EMS, and a large number of M_2 families are being screened at low humidity (about 50% RH) for the apomictic phenotype, where elongated siliques with seeds form in the absence of self-fertilization (Fig. 1). Based on the segregation for seed-set in the M_2 generation, grown from selfed individual M_1 plants (as individual families), two types of M_1 plants can be distinguished: type A giving progeny with seedless short siliques, and type B producing progeny that segregate in 1:2:1 for non-mutants:heterozygous for apomictic mutation: homozygous recessive for apomictic mutation (Fig. 1). The recessive nature of the mutation can be determined through inheritance studies by analysing the selfed progeny from individual sister plants that show seed-less short siliques (i.e. the heterozygotes for apomictic mutation). Seeds can be obtained from these plants by subjecting them to high humidity (90% RH) for a few days at flowering. These progenies will segregate in 3:1 for seed-less short siliques: seeded long siliques.

Recently, Peacock *et al.* (1995) reported that in 40 000 M_2 *pi/pi* (*pistillata*) plants derived from EMS treatment, eight mutants have been isolated in which partial development of seeds occurred without fertilization (*fis* mutants). In these mutants, i.e. *fis1, fis2*, and *fis3*, diploid endosperm developed normally to the point of cellularization. At a lower frequency, embryos degenerated without development beyond the globular stage. After pollination, the maternal embryos were

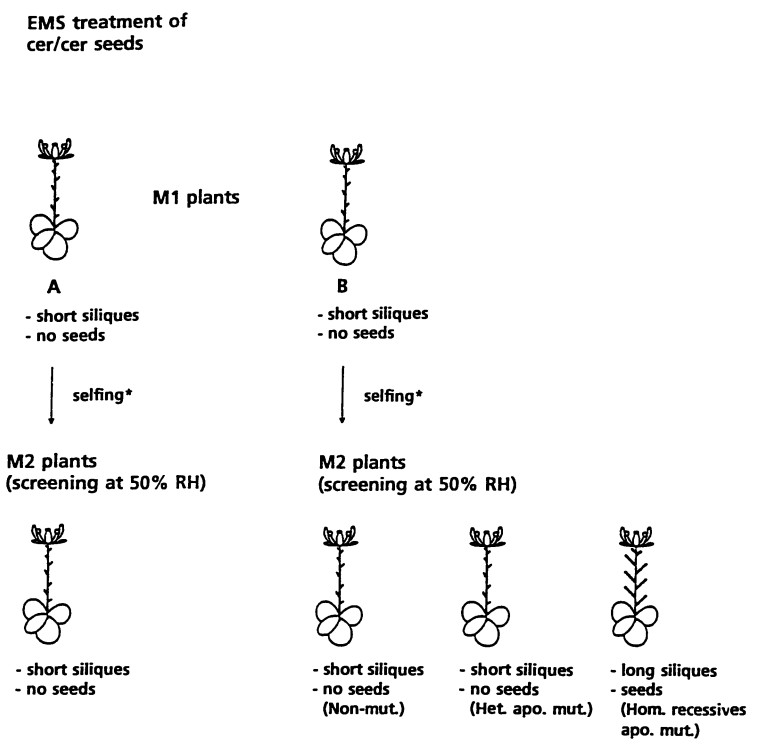

Figure 1. A schematic presentation of genetic screen to detect recessive mutations involved in apomictic seed production after ethyl methanesulphonate (EMS) treatment of seeds of *Arabidopsis thaliana eceriferum (cer)* genotypes. The homozygous *cer/cer* genotypes are conditionally male sterile, and do not produce functional pollen at low relative humidity (50% RH), and therefore no self-fertilization takes place. The siliques remain short and contain no seeds. However, at high relative humidity (90% RH), plants produce functional pollen and elongated siliques with seeds. Hence, this screening system enables detection of recessive mutations for embryo or endosperm development in the absence of self-fertilization (apomictic mutations). *Arabidopsis* plants with long or short siliques are shown in simple diagrams (abbreviated). A and B refer to two types of M_1 plants, which can be distinguished on the basis of segregation for seed-set in the M_2 generation. Type A gives M_2 progenies with seed-less short siliques, and type B produces progenies that segregate in 1:2:1 for non-mutants:heterozygous for apomictic mutations:homozygous recessive for apomictic mutations. *To obtain self-fertilized seeds for growing the M_2 population, the M_1 plants at flowering were subjected to 90% RH for a few days.

arrested at the torpedo stage, and the endosperm development to the cellular breakdown stage. The seeds from these mutants had a normal seed coat, but were inviable. Embryo rescue from these mutants resulted in the production of plants that gave rise to apomictic seed-set (completely filled siliques on inflorescence stem) (A.M. Chaudhury, personal communication). The *fis1* has been mapped on chromosome 1, *fis2* on chromosome 2 and *fis3* on chromosome 3, and cloning of *fis2* is in progress (which is now on a single YAC). Ohad *et al.* (1995) also screened about 50 000 EMS-mutagenized *cer* plants, and isolated 18 mutants, that

produced endosperm without fertilization (*fie*). In these, the ovule integuments and carpel differentiated into seed coat and silique respectively. The *fie* mutant allele was transmitted only by the female gametophyte. Embryogenesis seems to abort after fertilization even when the male contributes the wild-type allele, and therefore they concluded that the abortion was due to the effect of the female gametophyte.

Genetic Screen for the Detection of Dominant Apomictic Mutations after EMS Treatment

After EMS treatment of a large number of seeds, the M_1 plants are to be screened for seed-set at 50% RH. A dominant mutation for apomixis in the M_1 plant may produce elongated siliques with seeds in a portion of the plant or a shoot. In the dominant screen, using *pistillata*, Peacock *et al.* (1995) reported that after screening 15 000 M_1 plants derived from EMS treatment, 22 sectored inflorescences with dominant *Fis* phenotype were isolated. The mutant *fis4* appears to produce functional seeds autonomously, i.e. without pollination. The preliminary results indicated that *fis4* produces normal seeds with complete embryos (A.M. Chaudhury, personal communication).

Detection of Pseudogamous Apomictic Mutants

To detect pseudogamous apomictic mutants (dominant or recessive) and for visual identification of the apomictic seeds, it is highly essential to use a male parent containing dominant markers in pollen. Moreover, it needs a large-scale pollination (crossing) and seed (progeny) testing, and therefore, it is necessary to use a pollen parent that should facilitate an easy pollination (giving abundant pollen, convenient handling). In potato, embryo (seed) spot marker has been utilized for the detection of pseudogamy (Jongedijk, 1986).

Transposon Mutagenesis for the Isolation of Apomictic Mutants

Transposon Strategies

Transposons move from their chromosomal location, and induce mutations by inserting into genes and disrupting their structural integrity. The transposable elements transpose via excision and reintegration directly from DNA to DNA. Transposition is the result of an interaction of proteins (*trans*-acting factors) with the termini (*cis*-determinants) of transposable elements. These can be used as probes to clone genes that are mutated by insertion of this element. For this, the mutation caused by the transposon has to be identified by a genetic screening procedure. Subsequently, the gene can be isolated by cloning the DNA sequences flanking the transposable element insertion. This has been used successfully to isolate genes from *Zea mays*, *Antirrhinum majus* and other plant species, including

Arabidopsis and *Petunia* (Federoff *et al.*, 1984; Theres *et al.*, 1987; Martiensson *et al.*, 1989; Aarts *et al.*, 1993; cf. Walbot, 1992). It requires no prior knowledge about the nature of the product of the target gene, and depends only upon the expression of a mutant phenotype. Therefore, genes involved in flower, seed and other organ development, apomixis, as well as pathogen resistance, and certain physiological and biochemical processes can be isolated by transposon tagging.

Two approaches are currently used, i.e. targeted tagging and random tagging. The targeted tagging is generally used, when a mutant is already available that has been induced by other means than transposon insertion. A random tagging strategy is suitable for the isolation of a group of genes that belong, for example, to a developmental programme or to a particular biochemical pathway. This requires an extra generation of selfing to identify the putative transposon induced mutations. In a diploid plant species, such as *Arabidopsis* or *Petunia*, the mutant phenotype of a recessive mutation will become apparent in the M_2 generation upon selfing of the M_1 plants. Hence, new phenotypes can segregate in such selfed populations. Insertional mutagenesis with a known mobile DNA insert can generate mutations that are marked by a molecular tag. For the characterization of the mutants, co-segregation of a transposon copy with the mutant phenotype has to be established by analysing DNA from the parent, mutant and revertant plants in genomic Southerns. DNA sequences of the cloned mutant genes can then be used to isolate the corresponding wild-type allele. The tagged mutant gene can be cloned using the DNA insert as a molecular probe.

Transposon Mutagenesis in Arabidopsis

Transposon System

Transposon tagging in *Arabidopsis* can be employed using either one- or two-element systems. In a one-element system, an autonomous transposable element is used. In the two-element systems, the mobile transposable element is cloned into a selectable marker gene (e.g. for antibiotic resistance), blocking its expression. Excision of the mobile element (mediated by an autonomous element expressing transposase) restores the activity of the excision marker gene, which can be followed with a selective agent (e.g. antibiotic) in a phenotypic excision assay. In *Arabidopsis*, both one- and two-element systems have been developed using the maize transposons, i.e. *Ac-Ds* and *En-I*. However, as compared with the one-element system, the two-element systems were found to be more suitable for developing efficient transposon tagging systems (Aarts *et al.*, 1995). The *En-I* system revealed more favourable characteristics for developing the transposon tagging strategies than the *Ac-Ds* system. The *En-I* system was shown to be equivalent to the *Spm* system which has a different origin. The autonomous 8.3 kbp long *En/Spm* element (Pereira *et al.*, 1985, 1986) codes for two gene products *tnpA* and *tnpD*, which are required for transposition (Frey *et al.*, 1990; Masson *et al.*, 1989). The non-autonomous *I/dSpm* components are usually deletion derivatives of the *En/Spm* (Gierl *et al.*, 1989), which lack specific gene functions, and therefore cannot transpose by themselves. The *En-I* elements contain a 13 bp

inverted repeat at their termini and create a 3 bp target site duplication on insertion. The excision of the elements often leave behind a 'footprint', which may also generate a mutant or new allele.

Screening for Apomictic Mutations

The *En-I* system established for *Arabidopsis* cv. Landsberg erecta, and the conditional male sterile '*cer*' lines were used. The conditional male sterility trait of '*cer*' lines can facilitate the detection of apomictic mutants caused by transposable element insertions. The '*in cis En-I*' two-element system has a wings-clipped *En*-transposase under the control of the *CaMV 35S* promoter and a mobile *I* element inserted in the codogenic region of the *nptII* gene (Aarts *et al.*, 1995). The T-DNA contains a hygromycin resistance marker gene for the selection of transformants and progeny plants that carry the T-DNA locus. Active transpositions can be revealed in the progeny by DNA blot analysis with an *I*-specific probe. Several diverse transposon genotypes, each containing about 20-*I* elements and the *En* transposase, were crossed with *cer* lines (*cer1, cer6–2*) for the transfer of the conditional male sterility alleles to the transposon genotypes. Among the F_2 segregating population, homozygous *cer* genotypes (waxless plants) were selected and subjected to 90% RH for a few days to obtain selfed seeds for raising the F_3 generation. The F_3 plants containing transposable elements are to be selected based on resistance to hygromycin. A large population of the F_4 can then be screened for apomictic mutations by growing the plants at about 50% RH. To obtain a large number of independent transpositions which are distributed over the whole genome, and thus target the genes conferring apomixis, a continuous selfing can be performed, e.g. for two generations from the F_4 onwards, by subjecting the plants to 90% RH for some days at flowering, and the F_5 and F_6 population is screened for apomictic mutations.

Transposon Mutagenesis in Petunia

Transposon System

In *Petunia hybrida*, transposon lines are available that contain high copy numbers of transposable elements, which give rise to many unstable mutations in selfed progeny (Doodeman *et al.*, 1984; Gerats *et al.*, 1990). Moreover, *Petunia* is suitable for molecular analysis of ovule and flower development (Angenent *et al.*, 1995). *Petunia hybrida* has a two-element transposon system: a non-autonomous transposable element, designated defective transposable element *Petunia hybrida* 1 (*dTph1*), and an autonomous element carrying the transposase (activator *ACT1*) located on chromosome 1. The transposable element *dTph1* is 283 bp long (the smallest so far described) with an identical 12 bp sequence in an inverted orientation at each extremity. The terminal repeats show homology with terminal sequences of other transposable elements, e.g. *Tst1* from potato (Koster-Topfer *et al.*, 1990). The *Petunia* transposon system gives a high frequency of transposition (about 20% per selfed generation). Further, the line W138 used in this study

contains > 200 copies of *dTph1*, and gives rise to several types of unstable mutations in selfed progeny (Doodeman *et al.*, 1984). These include flower pigmentation (red–white sectors), plant and flower development, fertility and changes in the chloroplast system. In the absence of the autonomous element, the line W138 behaves as a stable recessive, and gives rise to white flowers. The majority of the excision events leaves behind a 'footprint' of 8 bp duplication of the border sequence of *dTph1* transposable element, showing sectorial flowers with red–white spots or sectors (Gerats *et al.*, 1990). This instability in the anthocyanin (*An*) locus has been used to determine the frequency of reversions, which is about 5%.

Conditional Male Sterile '*CHS*' System

A conditional male-sterile (self-sterile) line T1702 used for the detection of apomictic mutations is an anti-sense chalcone synthase (*CHS*) transformant of *P. hybrida*, which was produced by transformation of *P. hybrida* W115 through *Agrobacterium tumefaciens* strain LBA 4404, using the *chsA-chs* anti-sense VIB 176 construct (Ylstra *et al.*, 1994). In this line, flavonol biosynthesis is completely blocked due to the anti-sense inhibition of *chs* gene expression. The absence of flavonols due to this dominant mutation renders the plants self-sterile. Flavonols in either the anthers or the pistil are required for pollen tube growth and seed-set after self-pollination. The pollen of the anti-sense *chs* line, containing no flavonols in the exine, germinate and the tube grows to some extent, but later the tips burst open and stop further growth. The addition of flavonol aglycones (kaempferol, morin, quercitin, myricetin) or tobacco pollen in self-pollination, complements for the flavonol deficiency of T1702, and restores the pollen function, resulting in pollen tube growth and seed-set.

Screening for Apomictic Mutations

Various transposon genotypes, derived from the inbred *P. hybrida* line W138, were crossed with the conditional male-sterile line for the transfer of the conditional male-sterility alleles to the transposon genotypes to detect apomictic mutations. The F_1 genotypes are selfed by the addition of flavonols for obtaining selfed seeds. The externally applied flavonols (Ylstra *et al.*, 1994) complements *αCHS* activity, thus resulting in the pollen tube growth and the fertilization of ovules. Among the F_2 segregating populations, the plants showing homozygous dominant genotypes for *αCHS*, which contain transposable elements, are selected and selfed using flavonols. In F_3 and F_4, a large number of plants are screened for apomictic mutations. At this stage, no flavonols are used.

Conclusions and Perspectives

We have devised various genetic strategies to isolate mutants for apomixis after EMS and transposon mutagenesis in *Arabidopsis* and *Petunia*. *Arabidopsis* is a

proven experimental plant system for EMS and transposon mutagenesis for the isolation of genes involved in various plant processes. Because of the small genome size, limited repetitive DNA and world-wide collaboration for accessibility to mapped molecular markers, mapping lines, and sequence information, once the target gene is identified, it can be isolated relatively easily. Also, *Petunia* with a known transposon system and the availability of conditional male sterile antisense *αCHS* line, together with other advantages, is suitable for the induction and isolation of apomictic mutants. Peacock (1992) suggested that an *Esi* (embryo sac induction) gene is normally switched on in the megaspore after meiosis is completed, leading to the production of an embryo sac. In an apomictic plant, the *Esi* gene is switched on early in a group of nucellar cells, before the onset of meiosis. Each of these nucellar cells then responds to the *Esi* transcriptional factor to produce an embryo sac. Mutation of a sexual plant might result in the inactivation of a transcriptional repressor that normally suppresses an apomictic pathway, or might result in a mutant plant containing a transcriptional factor with a novel specificity, capable of inducing an apomictic pathway of reproduction (Chaudhury and Peacock, 1993; Koltunow *et al.*, 1995). Recently, Peacock *et al.* (1995) and Ohad *et al.* (1995) isolated several mutants in *Arabidopsis* after EMS mutagenesis, i.e. *fis* and *fie* mutants which seem to be associated with apomixis. The different *fis* mutants obtained after dominant or recessive screen have been mapped on different chromosomes, and investigations on cloning of genes are under way. Also, extensive mutagenesis research and genetic engineering for the isolation of genes controlling apomixis is being carried out in rice (Peacock, 1992; Khush *et al.*, 1994).

In addition, investigations for transfer by classical hybridization methods, of apomixis from wild to cultivated species in pearl millet (Hanna *et al.*, 1993, Hanna 1995a, b), maize (Savidan *et al.*, 1995) and wheat (Liu *et al.*, 1994) are progressing, though slowly due to problems of sexual incongruity and other genetic complications, i.e. the high polyploidy of the donor wild species. The recently developed technology, i.e. microprotoplast-mediated chromosome transfer (Ramulu *et al.*, 1996a, b) can be a useful alternative strategy for the transfer of the chromosome carrying the apomictic genes to the crop species. This can avoid tedious back-crosses followed by continuous selection, that are necessary in the case of classical plant breeding methods for eliminating the undesired chromosomes/traits and to retain the target chromosome. Yet, it is often difficult to eliminate the undesirable donor genes from the wild species and prevent 'linkage-drag' of 'hitch-hiking' genes (Sybenga, 1992).

Further, mutational and gene tagging approaches for the isolation of genes conferring the apomictic development would greatly facilitate the transfer of this trait to a much wider variety of crops. Tight linkage with molecular markers provides the opportunity to isolate such genes through chromosome walking. Transposable elements of maize, such as *En-I*, and of *Petunia*, i.e. *dTph1*, are being used in the present study to isolate genes for apomixis. Efforts are also under way in developing molecular maps necessary for map-based cloning of the apomictic gene(s) in *Pennisetum* and maize (Ozias-Akins *et al.*, 1993; Miles *et al.*, 1994; Savidan *et al.*, 1995). For genetic engineering of apomixis, however, there is a need for better understanding of mechanisms that trigger apomixis. More basic research is

needed to increase the knowledge on genetic regulation of apomixis. The isolation, cloning and characterization of genes for the different apomictic processes during megasporogenesis and female gametophyte development, such as disruption of meiosis, activation of nucellar cells and parthenogenetic development of embryo and endosperm, will greatly aid in gene transfer and manipulation of apomixis. These might also provide valuable information on the function and expression of apomixis at the diploid/polyploid levels, as well as on the differences, if any, between induced and natural apomixis.

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20. Induced Mutation in Fruit Trees

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Introduction

Genetic variation is essential for crop breeding. Induction of mutations is an effective method to enhance natural genetic resources. Induced and spontaneous mutations have played an important role in developing improved cultivars of various fruit trees as a supplementary method to conventional breeding. One of the earliest attempts to induce mutations was made by Stadler and Murneek (Stadler, 1930) by treating apple scions with X-rays. Thereafter, many mutants were induced in fruit trees, and a large number of reports were published. Out of induced and spontaneous mutants, some new genes such as self-compatible sweet cherry and Japanese pear, and thornless bramble were obtained, and have been widely used as breeding materials, as described in this review. Other examples include mutants such as russet-free fruit in apple, seedless *Citrus*, disease resistance in Japanese pear, and compactness in sweet cherry. These have been developed and released as new cultivars. Thus, mutation induction has been one of the important breeding methods especially in fruit trees which are vegetatively propagated, and have a high degree of heterozygosity.

Mutagenic Treatment

Chemical Mutagens

Since the mutagenic effect of mustard gas in *Drosophila* was reported by Auerbach and Robson (1946), several chemical mutagens have been used for the induction of mutants in many crops. The mutagenic effect of various chemicals has been investigated mainly by using seed-propagated crops. Among these, there have been some interesting reports that chemical mutagens such as ethylmethanesulphonate (EMS) and ethylene imine (EI) induce more mutations at some loci than physical mutagens, e.g. X-rays or γ-rays (Amano, 1972; Konishi, 1979). In fruit trees, the chemical mutagen must penetrate to the meristematic zones of scions, and the excess of chemical has to be removed after the treatment. Because of this difficulty, reports on mutagenesis with chemical mutagens have been fewer than those with physical mutagens (Micke *et al.*, 1985; Przybyla *et al.*, 1987; Privalov, 1986; IAEA, 1994). However, with the development of the recent technology of plant cell/tissue culture, the chemical mutagens have become more useful for *in-vitro* mutation breeding (Matsumoto and Yamaguchi, 1984).

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Physical Mutagens

UV, X-rays, γ-rays, β-rays and thermal neutrons have been used as physical mutagens by many researchers. Among these, the use of UV has been limited to cell suspensions and pollen grains because of its weak penetration. In case of β-rays, irradiation from radio-isotopes such as ^{32}P must reach the meristem cells of the scions (Ehrenberg and Granhall, 1952) because the penetration of such radiation in the plant tissue is weak. Extra care should be taken in handling such radio-isotopes, and the applicable safety regulations should be followed. This difficulty is also encountered with neutrons. Therefore, X-rays and γ-rays are the most convenient and easiest radiations for application and handling. Hence, it has been a natural trend that most induced mutations in fruit trees have been obtained with X-rays and γ-rays.

There are two irradiation methods with X-rays and γ-rays, i.e. chronic and acute irradiation. In the former, materials are irradiated with low dose rate but longer duration; this results in high survival but low mutation frequency, on the basis of accumulated radiation dosage. In the latter, irradiation is given for a shorter period, which gives low survival but higher mutation frequency compared to the former. Acute irradiations have been used by many researchers, because of the high construction costs, difficulty in maintenance and safety management of the chronic radiation facilities. Although the qualitative difference between the two methods is still not known for arboreal plants, γ-field irradiation is a convenient and effective method, as reported in Japan.

Radiosensitivity of the fruit trees depends on the species, cultivars and growth conditions of the material. In acute irradiation, LD_{50} or the lower dosage is preferred for vegetatively propagated crops, because mutants selected are occasionally accompanied by unfavourable mutations (Broertjes and van Harten, 1978). The organs and the stages used for irradiation depend on the purpose. With acute radiation, dormant scions are usually used to induce mutants for tree habit, fruit quality or resistance to diseases. When chronic irradiation is available, trees are planted in pots or directly in the irradiation field, and usually irradiated for years. The preferable dosage is difficult to predict, and depends on conditions such as dose rate, duration of irradiation and state of the material (Lapins *et al.*, 1969; Broertjes and van Harten, 1978). Examples of applicable acute and chronic irradiation dose, which allows new buds to sprout, are listed in Table 1 (Nishida *et al.*, 1967; Nishida, 1973; Donini, 1975). However, the values shown in Table 1 are only examples, because they may also vary with the irradiation conditions as described above (Lewis, 1949; Lapins *et al.*, 1969).

Chimaerism of Mutated Tissue and Management after Mutagen Treatment

Sectorial (Mericlinal) Chimaera

Mutations occur in a single cell of various tissues. The mutant cell in a meristematic region develops into a sectorial (mericlinal) chimaera by cell proliferation.

Table 1. Radiosensitivity of fruit trees

Species	Acute exposure LD_{50} dose(Gy)	Chronic exposure suggested dose (Gy/day)
Prunus persica	35 46[1]	0.15 0.45[7] 0.10–0.69[9]
P. avium	40 36[1]	–
P. cerasus	– 46–59[1]	–
P. domestica	40 46[1]	–
P. armeniaca	40 30–35[1]	–
P. amygdalus	– 44[1]	–
Pyrus pyriforia	–	– 0.50[7] 0.25–0.50[8]
P. communis	55 50–70[2] 50[6]	0.30 0.25–0.50[8]
Malus pumila	50 46–49[1] 52[3] 30[2] 50[6]	0.25 0.45[7] 0.25–0.50[9]
Rubus idoeus	50	–
Ribes nigrum	45	–
R. sativum	– 28[1]	–
Juglans regia	42 48[1]	–
Vitis vinifera	30	0.15 0.50[7]
Vaccinium angustifolum	– 59[1]	–
V. macrocarpa	– 64[1]	–
Corylus avellana	70	–
Diospyros kaki	–	– < 0.13[7]
Ficus carica	25 62[1]	–
Olea europaea	35	0.15
Citrus nobilis	55	–
C. sinensis	– 42[1] 40[4]	–
C. limonia	– 42[1]	–
C. paradisi	– 33[1] 50[5]	–
C. reticulata	– 49[1]	–
C. aurantium	– 42[1]	–

(Modified data based on Donini, 1975)

[1]Sparrow *et al.* (1968), [2]De Vries *et al.* (1970), [3]Lapins *et al.* (1969), [4]Spiegel-Roy and Padova (1973), [5]Hearn (1986), [6]Granhall (1953), [7]Nishida (1973): irradiated for about 1 year, [8]Nishida *et al.* (1967): irradiated for about 1 year, [9]Nybom (1961): irradiated for several years

For screening or detection of desired mutant character, we must wait until the mutant cell develops into a selectable size such as a shoot. For fruit characters, it may be a branch. To stimulate a mutant cell to develop into a selectable unit, cutting-back methods have often been applied (Kaplan, 1953; Bauer, 1957; Nakajima, 1965; Lapins, 1971; Donini, 1975). Irradiated shoots are designated as M_1V_1 propagation. The new shoot which develops from the M_1V_1 shoot, e.g. after cutting back, is designated as M_1V_2. The new shoot from M_1V_2 is called as M_1V_3, and so on. Shoots at M_1V_3 or M_1V_4 propagations are mostly free from sectorial (mericlinal) chimaeras. It is better to wait until M_1V_3 or M_1V_4 propagation to screen for the mutants.

Periclinal Chimaera

Three to five cell layers are formed in the meristematic zones in the terminal and lateral buds of many fruit trees. In vegetatively propagated materials, most bud sports reported have been periclinal chimaeras including polyploids (Einset, 1952; Pratt, 1967; Pratt *et al.*, 1972; Pereau-Leroy, 1974; Sanada *et al.*, 1987b). Replacement of cells between L-I and L-II layers is rare by conventional propagation methods, although occasionally such replacements have been observed between L-II and the other inner cell layers. This might be due to the fact that, in the L-I cell layer, periclinal cell division is very rare (Lapins and Hough, 1970). If a mutation is induced in L-I cells in the meristematic zone of a shoot apex of lateral or terminal bud, a shoot would develop into a periclinal chimaera with L-I mutant cells, leaving L-II and inner cell layers with original genotype, even in M_1V_3 or M_1V_4. The mutant which is the periclinal chimaera with the border plane between L-I and L-II layers seems to be stable as reported by studies of diploid–tetraploid periclinal chimaera (Einset, 1952; Pratt, 1967; Katagiri, 1976). There may not be many problems of instability due to chimaerism of this category in practical cultivation. However, the periclinal chimaerism in the mutant which has no mutant cells in the L-II cell layer cannot be used in cross-breeding, because sexual organs are derived from the L-II cell layer (Einset, 1952; Lapins, 1969; Sanada *et al.*, 1987b).

Phenotype Selection in Mutants

Agronomically important mutant characters such as compactness in tree habit, fruit skin colour or russetness, can be selected visually. Many such mutants have been released as new cultivars after field evaluation (Table 2). Usually, selections were performed at M_1V_3 or M_1V_4 propagations after scions had been irradiated by X-rays or γ-rays.

Apple *(Malus pumila* Mill. var. *domestica* Schneid., *2n = 34, 51, 68)*

Many researchers have focused on mutations for compact and spur type tree habit, improved skin colour or russet-free skin in fruit appearance, and resistance to diseases. LD_{50} in acute irradiation of dormant scions was 50 Gy (Lapins, 1965a; Lapins *et al.*, 1969; Donini, 1975); the applicable irradiation dose ranged between 30 and 70 Gy (Lapins, 1965a; Lapins *et al.*, 1969; De Vries *et al.*, 1970; Donini, 1975). Lapins (1965a) reported that the frequency of induced mutants for compactness in 'McIntosh' was 3% (37.5 Gy) or 5% (50 Gy) of surviving trees. Many compact type mutants including spur type were selected (Lapins, 1965a; Decourtye, 1970; Visser *et al.*, 1971; Poll, 1974; Ikeda, 1977; Lacey and Campbell, 1979; Lacey, 1982; Zagaja *et al.*, 1982). Because of dwarf rootstocks such as 'M' and 'MM' in apple, the compact type mutants were not released as

Table 2. Cultivars developed through induced mutations[1]

Fruit	No. of released cultivars[2]	Mutagens				Mutant character
		γ	X-ray	Thermal neutrons	Chemical mutagens	
Apple	10	8	1	0	1	Compact tree, early-maturing, red colour fruit skin, russet-free fruit skin, variegated leaf
Pear	3	3	0	0	0	Disease resistance
Peach	3	3	0	0	0	Early-maturing
Sweet cherry	5 + (3)[3]	2	3	0	0	Compact tree, self-compatible
Sour cherry	4	3	1	0	0	Compact tree, fruit set
Plum	1	1	0	0	0	Early-flowering
Apricot	1	0	0	1	0	Early-maturing
Bramble	1	0	0	0	1	Disease resistance
Currant	2	1	1	0	0	Early-maturing, erect growth
Grape	1	1	0	0	0	–
Pomegranate	2	2	0	0	0	–
Fig	3	1	2	0	0	Mosaic leaf
Sea buckthorn	1[4]	0.5	0	0	0.5	–
Citrus	5	3	0	2	0	Seedless, red colour of fruit and juice
Olive	1	1	0	0	0	Short branch
Loquat	1	1	0	0	0	–
Total	44 + (3)[3]	30.5	7	3	2.5	

[1]The information is based on *Mutation Breeding Newsletter* and *Mutation Breeding Review*, published by IAEA.

[2]*Apple*: Belrene, Blackjoin BA 2 520, Courtagold, Coutavel, Golden Haidegg, Lysgolden, McIntosh 8F-2-32, Mori-Hou-Fu 3A, Shamrock, Dovar. *Pear*: Gold Nijisseiki. The other two are under processing for registration. *Peach*: Magnif 135, Plovdiv 6*, Fukuekubo. *Sweet cherry*: Burlat C1, Compact Lambert, Compact Stella 35B11, Ferrovia spur, Nero II C1, (Lapins)[3], (Stella)[3], (Sunburst)[3]. *Sour cherry*: Karlik Samorodka, Polukarlik Orlovskoi, Polukarlik Turgenevki, Plodorodnaya. *Plum*: Spurdente-Ferco. *Apricot*: Early Blenheim. *Bramble*: Kolokol'chik. *Currant*: Westra, Burga. *Grape*: Fikreti**. *Pomegranate*: Khyrda**, Karabakh**. *Fig*: Golden King, Golden Princess, Bol**. *Sea buckthorn*: Zyrianka[4]. *Citrus*: Star Ruby, Rio Red, Hongju 418, Hongju 420, Xue Gan 9-12-1. *Olive*: Briscola. *Loquat*: Shiro-mogi** (*, pollen irradiation; **, seed irradiation)

[3]Three cultivars were bred by cross-breeding using mutants as parent.

[4]Seed was irradiated with γ-rays and seedlings were treated with chemical mutagen.

new cultivars. Only 'Courtagold' from 'Golden Spur' and 'Courtavel' from 'Starking Delicious' were released as registered cultivars (IAEA, 1987). Recently, the columnar tree shape which is one of compact types has become an important breeding objective. This was selected as a spontaneous mutant, 'Wijcik' from 'McIntosh'. In contrast to 'Wijcik' which has a single dominant gene controlling the columnar tree shape (Lapins, 1976), the compactness is determined by

polygenes because continuous variation in growth types is observed in the mutant progeny (Lapins, 1974a).

Red skin mutants have been induced in many major cultivars (Bishop, 1954, 1959; Decourtye, 1970; Lapins, 1972; Yoshida *et al.*, 1981). Some induced mutants such as 'Morihou-Fu 3A' from 'Fuji' (Yoshida *et al.*, 1981) irradiated with 30 Gy γ-rays (1.5 Gy/h) have been released. Although, these induced mutants with more pronounced red skin colour can be easily selected, these characters in major cultivars would not be suitable as the target of mutation induction, because the spontaneous mutants were also frequent and easily selected by growers who own many trees. Actually, spontaneous red mutants were found in many important cultivars, e.g. 'Delicious', 'Fuji', 'Jonagold', 'Gala', 'Tsugalu' and 'Jonathan'. Fruit skin colour is considered to be controlled by a suppressor, and the colour change occurs from mutation in the suppressor gene (Bishop, 1957). These mutants are useful in cross-breeding, if the mutant cells are in the L-II cell layer.

The other fruit skin character, russet-free, was selected from irradiated shoots of 'Golden Delicious' (Decourtye, 1967, 1970), and it was released as 'Lysgolden' in 1970. 'Golden Haidegg' was also selected as russet-free from 'Golden Delicious', irradiated with 50 Gy γ-ray at a dose rate of 20 Gy/min, and released in 1984 (Strempl and Keppl, 1988; Brunner and Keppl, 1991). 'Golden Haidegg' seems to have more attractive skin colour, taste and cold-storability of fruit than 'Lysgolden' (Strempl and Keppl, 1988; Brunner and Keppl, 1991). 'Lysgolden' and 'Golden Haidegg' are the two cultivars, which have been recommended by the European Community to replace 'Golden Delicious' (Brunner and Keppl, 1991).

Mutants with early maturity and large fruit size have been also reported. An early-maturing mutant with deeper colour and larger fruit from 'Reine des reinettes' was obtained after treatment with 1% aqueous solution of EMS. It was released as 'Belrene' in 1970 (Micke *et al.*, 1985). An early-maturing mutant was also induced in 'Fuji', although the fruit skin was not attractive. Another early-maturing spontaneous mutant of 'Fuji' was released as 'Yataka'.

European Pear (Pyrus communis L., *2n = 34, 51, 68) and Japanese Pear (P. pyrifolia* Nakai, *2n = 34)*

Many researchers have focused on compactness and spur type, skin colour and resistance to diseases in pear. The LD_{50} of acute irradiation to dormant scions of the European pear seems to be 55 Gy (5–6 Gy/h) (De Vries *et al.*, 1970; Donini, 1975), and the practical irradiation dosage seems to be between 30 and 50 Gy (Decourtye, 1970; De Vries *et al.*, 1970). Several compact type mutants were induced in the European pear (De Vries *et al.*, 1970, Visser *et al.*, 1971). The frequency of induced compact mutants was low, only about 0.5% (Visser *et al.*, 1971). Besides the compact type, mutants with green-yellow-striped skin fruit were also obtained (Granhall, 1953).

In Japanese pear, several mutants resistant to black spot disease could be induced by chronic irradiation with doses of 0.12–0.15 Gy/day γ-rays (Sanada

et al., 1987a, 1988; Sanada, 1988a,b). 'Nijisseiki', which has been a leading cultivar in Japan for almost a century, is susceptible to black spot disease, caused by *Alternaria alternata*, Japanese pear pathotype (Tanaka, 1933; Nishimura *et al.*, 1978). The susceptibility is controlled by a single dominant gene and 'Nijisseiki' is heterozygous for this allele (Kozaki, 1973). Induced mutants showed intermediate resistance to the disease (Sanada *et al.*, 1988, Sanada, 1988b). The intermediate resistance of this mutant, 'γ-1-1' could be explained as follows: one possibility is formation of periclinal chimaera which consisted of cells of the original 'Nijisseiki', in the L-I layer and the resistant mutant cells in the L-II layer as shown by histological studies where the epidermal layer is affected by the pathogen (Sanada, 1988b). The other possibility is that the intermediate resistance was induced in the L-II layer, because the intermediate resistance in the mutant, 'γ-1-1' was transmissible to the F_1 seedlings (Sanada *et al.*, 1994). The mutant was released in Japan as 'Gold Nijisseiki' in 1991 (Kotobuki *et al.*, 1992; Sanada *et al.*, 1993).

A self compatible mutant, 'Osa-Nijisseiki' ($S_2S_4^{sm}$) was selected as spontaneous stylar mutant from 'Nijisseiki' (S_2S_4) (Sato *et al.*, 1992). S-glycoproteins associate with the self-incompatibility in the Japanese pear, and the level of S_4 glycoprotein in the self-compatible 'Osa-Nijisseiki' is much lower than the original self-incompatible 'Nijissaeiki' (Sassa *et al.*, 1993; Hiratsuka *et al.*, 1995). The S-glycoproteins were identified as S-RNases (Sassa *et al.*, 1993, 1996a). After cDNA analysis, Sassa *et al.* (1996b) suggested that the self-compatibility in the mutant is due to the deficiency of the S_4 gene.

Sweet Cherry (Prunus avium L., *2n = 16, 24, 32) and Sour Cherry (P. cerasus* L., *2n = 32)*

Self-compatibility is an important character desired in sweet cherry for which genes are not known, although self-compatibility genes have been used for cross-breeding in apple ('Megumi': Saito *et al.*, 1978) and Japanese pear ('Osa-Nijisseiki'). In sweet cherry, pollen mother cells were irradiated with X-rays before the expression of S genes, and induced self-compatible pollens were searched by self-pollination (Lewis, 1949, 1956; Lewis and Crow, 1954). The required dose was about 8 Gy. Out of 106 seedlings obtained, 42 were investigated further, of which four were fully self-fertile (Lewis and Crow, 1954). The same technique was applied to apple and pear, and several self-fertile seedlings were obtained (Lewis and Crow, 1954). The induced self-fertile genes in sweet cherry were stable, and about 50% of seedlings were self-compatible, when such trees were used as one of the parents. Self-compatible mutants were also used for cross-breeding, and many self-compatible cultivars, such as 'Stella', 'Lapins' and 'Sunburst' have been released in Canada (Lapins, 1974c; Maluszynski *et al.*, 1992). The self-compatible 'Stella' was analysed to be S_3S_4' (Lapins, 1973), where S_4' is the mutant form of S_4 which is well known to have lost the self-incompatibility in pollen. Analyses of RNases associated with self-compatibility were reported by using extracts from styles. The style extract from S4′ was not

significantly different from that of S_4 in the sweet cherry mutant which has lost the activity in pollen (Boskovic and Tobutt, 1996). The result was in contrast with the pear which has lost the style activity (Sassa *et al.*, 1993; Hiratsuka *et al.*, 1995).

Several compact type mutants have been released as cultivars in sweet cherry and sour cherry in which desirable dwarf rootstock was not available. The LD_{50} of acute irradiation for dormant scions seemed to be 50 Gy (4.4–10 Gy/h) (Donini, 1975) and the practical dose between 25 and 50 Gy (Lapins, 1971; Donini, 1975; Zagaja *et al.*, 1982). The 5th to 10th buds on primary shoots from M_1V_1 were preferred for screening sectorial chimaera, and many compact type mutants were obtained (Donini, 1975; Lapins, 1971; Zagaja *et al.*, 1982). Compact type mutants were obtained with high frequency from M_1V_3 shoots, grown from 5th to 10th buds on M_1V_2 (Lapins, 1971). Several induced compact mutants, e.g., 'Compact Stella' (Lapins, 1974b), 'Compact Lambart' in Canada (Lapins, 1965b), 'Burlat C1' and 'Nero II C1' (IAEA, 1988) and 'Ferrovia spur' in Italy (IAEA, 1996) were released.

In sour cherry, many compact mutants were induced in 'Orel Early', 'Turgenevka' and 'Samorodka' after γ-irradiation, and 'Polukarlik Orlovskoi Rannei', 'Polukarlik Turgenevki' and 'Karlik Samorodka' were released as new cultivars in the USSR (IAEA, 1981).

Peach (Prunus persica (L.) Batsch., *2n = 16), Plum (P. domestica* L., *2n = 48), Apricot (P. armeniaca* L., *2n = 16, 32) and Almond (P. amygdalus* Batsch., *2n = 16).*

In peach, the LD_{50} of acute irradiation for dormant scions was 35 Gy (6.12–20 Gy/h) (Donini, 1975) or 46 Gy (Sparrow *et al.*, 1968). The LD_{50} depended on the dose rate, being 150 Gy at 0.12 Gy/min and 37 Gy at 8 Gy/min for summer buds (Lapins *et al.*, 1969). The practical dose of acute irradiation for dormant scions ranged between 10 and 50 Gy (Donini, 1975). In peach many agronomically important characters, such as nectarine type (pubescent skin/glabrous; *G/g*), melting flesh (melting/non-melting; *M/m*), flesh colour (white/yellow; *Y/y*), resistance to nematode (*Meloidogyne incognita* resistant/susceptible; *Mi/mi*) and dwarfness (normal/brachytic; *Dw/dw*) are determined by single genes (Hesse, 1975). Many mutants, such as nectarine type (Donini, 1975), early or late fruit maturity (Hough and Weaver, 1959; Nishida, 1973; Donini, 1975), flesh firmness, flesh colour, freestone (Hough and Weaver, 1959), large fruit size, deep red colour of fruit skin and high yield (Micke *et al.*, 1985) could be induced. Of these mutants, 'Magnif 135' was released as a new cultivar with large fruit size, early maturity and red colour of fruit skin. 'Plovdiv 6', which was selected after pollination of pollen irradiated with γ-rays, was released as a new cultivar with increased yield, large fruit size and good quality (Micke *et al.*, 1985). Recently, an early-maturing mutant was induced in 'Akatsuki', irradiated with 30 Gy (2.5 Gy/h) γ-rays, and was released in 1995 as 'Fukuekubo', which matures about 10 days earlier than the original cultivar.

In plum (*P. domestica*), one mutant with early flowering and improved fruit setting was released as a new cultivar, 'Spurdente-Ferco' (IAEA, 1990). In apricot (*P. armeniaca*), one mutant induced with thermal neutrons, matured 1 week earlier than the original cultivar, and was released as 'Early Blenheim' (Micke *et al.*, 1985). In almond (*P. amygdalus*), a mutant with 12 days delayed flowering which escapes frost damage was induced with 30 Gy γ-rays (IAEA, 1988).

Citrus (*Citrus* spp., *2n = 18, 27, 36)*

Many citrus species are propagated by nucellar embryos and scions grafted on rootstocks. Both scion and seed are often used in mutation induction. However, nucellar embryos require long duration to reach flowering age, when used as planting material. Therefore, scions have been often used where a change in fruit character is the purpose of mutation induction. Several research papers have been published on seedlessness, which is an important character in citrus.

The irradiation of nucellar embryos at floral stage is useful to eliminate periclinal chimaeras (Ikeda, 1976). In an experiment, potted trees of *Citrus sunki* were irradiated with 20 or 40 Gy (1 or 2 Gy/h) γ-rays at three different floral stages and pollinated with *Poncirus trifoliata* which has a single dominant gene controlling the trifoliate character; hence the nucellar embryos could be distinguished from the hybrid embryos. Solid mutants were obtained by cutting back the seedling which induced the formation of adventitious buds. Radiosensitivity of mature seed was lower than that of the floral stage embryos, and LD_{50} was between 80 and 100 Gy in mature seeds of *C. sinensis* (Spiegel-Roy and Padova, 1973). In China, polyembryonic seeds of cv. 'Jin Cheng' (*C. sinensis*) were irradiated with 100 Gy γ-rays. Two mutants which were almost seedless were selected 15 years after irradiation, and released as new cultivars, 'Hongju 418' and 'Hongju 420' (Chen *et al.*, 1991). In these clones, there was no variation in chromosome number; however, univalents and multivalents could be observed at meiosis in PMC (Chen *et al.*, 1991). A seedless mutant clone was also selected among seedlings of grapefruit (*C. paradisi*) cv. 'Hudson' which was irradiated with thermal neutrons, and released as 'Star Ruby' (Micke *et al.*, 1985; Hensz, 1991).

A spine-free mutant was also induced in nucellar seedlings of *C. sunki*, irradiated with 40 Gy γ-rays (Kukimura *et al.*, 1976). To induce mutants with seedlessness or flesh/skin colour of fruits, many researchers have irradiated scions with γ-rays. The LD_{50} was 55 Gy in summer buds of *C. nobilis* (10 Gy/h) (Donini, 1975), between 40 and 80 Gy in *C. sinensis* (Sparrow *et al.*, 1968; Spiegel-Roy and Padova, 1973), and 33–50 Gy in *C. paradisi* (Sparrow *et al.*, 1968; Hearn, 1986). One completely seedless mutant was selected out of 600 M_1V_3 plants of lemon (*C. limon*), cv. 'Eureka', irradiated with 60 Gy γ-rays (Spiegel-Roy *et al.*, 1985). Another completely seedless mutant was selected among 120 M_1V_2 plants in lemon cv. 'Israeli Villafranca', irradiated with 50 Gy γ-rays (Spiegel-Roy *et al.*, 1990). Spiegel-Roy and Vardi (1990) reported that in addition a seedless mutant was induced in cv. 'Minneola' tangelo. These three mutants had sterile

ovules and fertile pollen. Five 'near seedless' mutants were selected among 23 M_1V_2 plants of grapefruit cv. 'Foster', irradiated with 50 Gy γ-rays. Hearn (1986) reported that the bud irradiation method was preferable because the mutants could be selected in significantly less time than seed irradiation. Wu *et al.* (1986) selected 15 seedless mutants from five cultivars, irradiated with 80 Gy of γ-rays. A mutant with deep red colour of flesh and juice was induced in cv. 'Ruby Red' (*C. paradisi*) irradiated with thermal neutrons, and released as cv. 'Rio Red' in 1984 (Hensz, 1991; IAEA, 1991).

Other Fruit Trees: Grape (Vitis), Currant (Ribes), Olive (Olea), Raspberry (Rubus), Sea Buckthorn (Hippophea)

The reported LD_{50} in grape (*Vitis vinifera*) was 30 Gy γ-rays (2.35–5.50 Gy/h) (Donini, 1975), and the practical dosage of acute irradiation to dormant scions was about 30 Gy (Shimotsuma, 1962; Donini, 1975). Some mutants with short internodes, early fruit maturity, seedlessness and resistance to berry fall were induced in *V. vinifera* cv. 'Bonarda', and 'Regina dei Vigneti', irradiated with γ-rays (Donini, 1975). In muscadine grape (*V. rotundifolia*), a large fruit bud sport which was tetraploid, was induced with irradiation of 27 Gy γ-rays (Fay, 1963).

In blackcurrant (*Ribes nigrum*), the reported LD_{50} was 45 Gy γ-rays (6.2 Gy/h) (Donini, 1975). The suggested dose of acute irradiation for dormant scions was between 20 and 30 Gy (Bauer, 1957; Donini, 1975). A mutant with strong erect habit was induced in 'Westwick Choice', irradiated with 15 Gy γ-rays, and a new cv. 'Westra' was released in Germany (Micke *et al.*, 1985). A mutant with 1 week earlier maturity was selected from 'Noire de Bourgogne', irradiated with γ-rays, and was released as cv. 'Burga' (IAEA, 1987).

In raspberry (*Rubus idaeus*), the reported LD_{50} was 50 Gy γ-rays, and for mutation induction γ-ray doses ranging between 20 and 60 Gy (6.2 Gy/h) were suggested for dormant scions (Donini, 1975). However, there are no reports on induced mutants with γ-rays irradiation. A mutant with disease resistance and winter hardiness was induced in cv. 'Karnaval' treated with 0.025% ethyl nitrosourea (ENH), and was released as cv. 'Kolokol'chik', in the USSR (IAEA, 1994). In blackberry (*Rubus* sp.), thornless type is a very important character, and many thornless mutants were known. A dominant gene for thornless was identified in a spontaneous mutant cultivar, 'Austin Thornless' (Jennings, 1984).

In olive (*Olea europaea*), the reported LD_{50} is 35 Gy γ-rays, and for mutation induction in dormant scion or rooted scion the dose ranged between 10 and 60 Gy. Some mutants with short internodes, spur type and higher oil content were selected (Donini, 1975, 1982). A new cultivar, 'Briscola', with 50% reduced plant height, was induced in cv. 'Ascolana Tenera', irradiated with 40 Gy γ-rays, and released in Italy. The reduced height is desirable in harvesting (IAEA, 1982).

Sea buckthorn (*Hippophea rhamnoides* L.), which originates in Central Asia, is a dioecious tree belonging to the family Elaeagnaceae, and has been cultivated in Russia and Central Asia as a medicinal oil plant. The sea buckthorn has been

brought into cultivation recently. Therefore, breeding of cultivars is not so well developed. In order to obtain high-yielding mutants, the seeds of cv. 'Altai' were treated with 150 Gy, then the seedlings were treated with 0.01% nitrosomethyl urea. A mutant with large fruits, high yield and an increased content of oil, sugar and carotenoids was selected (Privalov, 1986).

Screening of Mutant with Phytotoxins and *In-vitro* Culture Methods

Use of Phytotoxins

The development of selection methods is essential for screening of disease-resistant mutants. Powdery mildew, which is an important disease of apple, is caused by *Podosphaera leucotricha*. Resistant mutants to this disease were selected from γ-irradiated shoots of 'McIntosh'; the wide range in resistance, from strong resistant to nearly susceptible clones, was recognized (McIntosh and Lapins, 1973). Several dominant genes are known for resistance to the disease in the wild species (Knight and Alston, 1968; Korban and Dayton, 1983; Gallott *et al.*, 1985). Resistance in the induced mutants should be identified by comparing with the resistance in the wild types. For this purpose, a stable evaluation method, such as pathotoxin screening, is required. Unfortunately, such a toxin is still to be found for this pathogen, in addition to the difficulty of maintaining various strains of the pathogen.

For screening of mutants resistant to black spot disease in Japanese pear, Sanada (1988a) developed a selection method using host-specific, AK-toxin I (Nakashima *et al.*, 1982, 1985). The toxin was adjusted to concentration at which the resistant mutant, 'γ-1-1' could be selected. Leaf discs obtained by punching from the 4th leaf were incubated on a layer of two filter papers, moistened with 0.1 ppm AK-toxin I for 2 days at 25°C. It was possible for one person to screen about 500 shoots per day. The mutation frequency for plants grown in the γ-field, Ohmiya-machi, Japan, was estimated at 1.3×10^{-3} of 0.12 Gy/day and 1.6×10^{-3} of 0.18 Gy/day from chronic irradiation (Sanada, 1988a).

This screening methods were extended to the black spot disease-susceptible cvs. 'Shinsui' and 'Osa-Nijisseiki' (the latter is a spontaneous self-compatible (S^{sm}_4) mutant of 'Nijisseiki') by using 80 Gy of acute γ-irradiation (Murata *et al.*, 1994). The disease-resistant mutant of cv. 'Shinsui' is under registration for release as a new cultivar. The disease-resistant mutants from 'Osa-Nijisseiki' were also selected by the same method from the materials irradiated chronically, 13.9 mGy/h (Masuda *et al.*, 1997). One of them is also under registration for release. All the induced mutants resistant to black spot disease in Japanese pear cvs. 'Nijisseiki', 'Osa-Nijisseiki' and 'Shinsui' showed intermediate resistance like 'γ-1-1'. The intermediate resistance might be due to the periclinal chimaera, as mentioned before. If that was the case, fully resistant mutant clones would be expected from regeneration of the plants through cell culture of the mutant.

In-vitro *selection*

Tissue culture techniques are now well developed, and can be used for *in-vitro* selection of mutants. In many fruit trees, it is easy to produce many shoots in a short duration by the micropropagation technique (Sona and Erez, 1980; Lyrene, 1981; Singha, 1982; Qi-guang *et al.*, 1986). Thus, it is possible to obtain many shoots of M_1V_3 or M_1V_4 plants after irradiation in a short time and throughout the year (Donini, 1982; Sanada, 1983; Tabira *et al.*, 1993; Masuda and Yoshioka, 1996). Sanada (1983) carried out *in-vitro* γ-irradiation of shoots of apple rootstock, 'M. 27', and found that the shoots could survive up to 120 Gy at the dose rate of 5 Gy/h, although the propagation rate in 120 Gy was reduced to less than half of that of the control in *in-vitro* subcultured M_1V_2. Several *in-vitro* methods of mutant selection have been reported, e.g. compact type by Lane and Looney (1982), rooting ability by Sanada (1983) and resistance to disease by Tabira *et al.* (1993) and Masuda and Yoshioka (1996). A mutant, resistant to black spot disease, was selected among 672 plants of 'Osa-Nijisseiki' of Japanese pear propagated *in-vitro* after γ-irradiation with 80 Gy (5 Gy/h). The mutant was obtained by incubating the leaves of propagated plants with host-specific AK-toxin I of the pathogen (Tabira *et al.*, 1993).

An apple mutant resistant to *Alternaria* blotch disease was selected (Masuda and Yoshioka, 1996). *Alternaria* blotch is a serious disease of apple in Japan, and is caused by *Alternaria alternata* apple pathotype. The susceptibility is determined by a single dominant gene (Saito and Takeda, 1984) as in the case of Japanese pear. The susceptible cultivar 'Indo' is heterozygous for the locus. The mutant was selected out of 3002 M_1V_4 shoots propagated from γ-irradiated shoots with a dose of 80 Gy (5 Gy/h) *in vitro*. The mutant was screened by incubation of leaves of micropropagated plants with the host-specific toxin, Alternarioride (Okuno *et al.*, 1974). The induced mutant showed intermediate resistance as in the case of 'γ-1-1' of Japanese pear (Masuda and Yoshioka, 1996).

Regeneratation of plants from callus, shoot, leaf segment and young embryos was reported in *Citrus* spp. (Rangan *et al.*, 1968; Kochba and Spiegel-Roy, 1973), *Prunus* spp. (Mehra and Mehra, 1974; Matsuda *et al.*, 1983; Hammerschlag *et al.*, 1985), kiwi fruit (Harada, 1975), grape (Favre, 1977; Hirabayashi and Akihama, 1982), pear (Janick, 1982; Ochatt and Caso, 1986) and apple (Eichholtz *et al.*, 1979; Tsukahara *et al.*, 1985). Embryogenic callus and somatic embryogenesis was obtained from unfertilized ovules in *Citrus sinensis* (Kochba *et al.*, 1972; Spiegel-Roy and Kochba, 1973; Button *et al.*, 1974). By using these techniques, mutation induction *in vitro* has been developed in many fruits, especially in *Citrus* and related genera. When embryogenic calli were irradiated with 5–320 Gy (31 Gy/h), the doses above 240 Gy decreased callus growth, were lethal at 320 Gy, and stimulated embryoid formation at 160 Gy (Spiegel-Roy and Kochba, 1973). In *Poncirus triforiata*, a salt-tolelant clone was selected from adventitious embryos generated from tissues between shoot and root. The materials were screened with 0.2% NaCl solution after mutagenic treatment with 0.3% EMS

(Matsumoto and Yamaguchi, 1984). Embryogenic lines tolerant to NaCl or 2,4-D were selected on medium containing 0.5% NaCl or 10^{-5} M 2,4-D after treatment with 80–160 Gy (0.8 Gy/h) (Kochba and Spiegel-Roy, 1982).

Omura *et al.* (1987) selected many morphological mutants of pomegranate (*Punica granatum* L. cv. 'nana') with changed leaf shape and tree habit which were regenerated from leaf segments irradiated *in vitro* with γ-rays. The doses between 320 and 640 Gy (2.5 Gy/h) reduced callus formation by 50% of the control, and doses between 160 and 320 Gy caused 50% reduction in adventitious bud formation.

Tissue culture techniques are promising for selection of mutants; however, not many mutants have been selected by using these techniques. Appropriate stress needs to be imposed for effective selection of mutants from embryogenic cultures or other regenerative systems. Recently, *in-vitro* screening methods for resistance to bacterial spot (*Xanthomonas campestris* pv. *pruni*) and nematodes (*Meloidogyne incognita*) have been developed in peach (Hammerschlag, 1988; Huettel and Hammerschlag 1986; Hashmi *et al.*, 1994). The development of an *in-vitro* screening method is needed for the effective selection of useful mutants.

Future Prospects and Conclusions

Mutation techniques have proven to be an effective breeding method, especially for vegetatively propagated crops and fruit trees. In most cases, heterozygosity of the genotypes is desirable for heterotic vigour. Such heterozygosity of genotypes often give good results with mutation breeding. Advances in molecular genetics established that genes are pieces of DNA in which the sequence of bases determines genetic information (Klösgen *et al.*, 1986). Mutations may be viewed as disturbances in the meaningful (exon) sequences. Such disturbances inactivate or modify the produced enzyme. This may explain why recessive mutations predominate after mutagenic treatment. When disease susceptibility is determined by a dominant gene, as in the case of Japanese pear, and the plant is heterozygous for the gene, induced mutagenesis will be the best method to obtain disease resistance. Mutation techniques had been considered as a black-box which may or may not produce the desired types. Now the results can be foreseen, where the genetic basis of the phenotype is known to the breeder. Studies in transposable DNA sequence developed a unique way to induce mutations by gene-splitting using T-DNA in *Arabidopsis* (Feldmann *et al.*, 1989) and other plants. Splitting of the gene inactivates the gene function resulting in a recessive mutation. The mutant would be a precious resource to clone the gene by tagging of T-DNA, no doubt (Shimamoto, 1994). Gene-splitting by introduced short DNA sequence may induce complete disorder of the gene, but ordinary induced mutation may offer a base pair exchange, resulting in a mild leaky mutation, if an appropriate mutagen is used (Amano, 1972, 1985; Sano *et al.*, 1985; Yatou and Amano, 1991). The stability of the modified gene must be carefully examined. At the present stage, the cost and safety of the transgenic methodology may prove problematic to use

directly in fruit tree breeding. Another molecular technique of antisense sequence may be promising where multiple copies of a gene exist in a genome and when they have some common sequence in them, because induced mutation techniques cannot overcome all of the multiple copies, e.g. 10 copies of an allergen storage protein in rice grain (Sato, 1996; Tada, 1996). On the other hand, if a wanted character is controlled by a dominant or metabolically active gene, mutation induction may not be a good idea. If a breeder wants to retain the other characteristics, or the original genetic background of a cultivar, cross-breeding may not be appropriate either. In such cases, perhaps gene insertion through transgenesis may be a suitable method.

Mutation and molecular techniques are complementary methodologies in crop improvement. The technologies of mutation induction and recombinant DNA should not be considered as rival methodologies. The choice of the method should be based on the improvement objective. Effective usage will depend on the purpose and genetic nature of the desired character, whether to metabolically activate a gene or inactivate a gene. Breakthrough in the current problems of cost and bio-safety will give us a complete set of gene-handling techniques. Even so, conventional breeding will still remain the major breeding methodology in crop improvement using new and/or mutated genes, although mutation breeding and molecular techniques will be very effective where only a few characters need to be improved.

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21. Induced Mutations and Somaclonal Variation in Sugarcane

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Mutation breeding as a methodology for crop improvement is based on the possibility of altering genes by exposing vegetative propagules, gametes or seeds to chemical and physical mutagens. The methodology is considered particularly useful in improving vegetatively propagated crops, as the mutated character can be maintained through asexual propagation. Many cultivars of this group of crops represent unique adaptive gene complexes which when passed through a sexual cycle for improvement of specific characters are bound to be irrevocably lost. All sugarcane cultivars grown today are highly heterozygous and complex polyploids, produced through interspecific hybridization involving three or four species of *Saccharum*. Consequently a considerable time is taken to improve sugarcane varieties for specific desirable traits through a conventional breeding programme. In this context, sugarcane breeders explored the possibility of mutation breeding to rectify specific defects or to improve a specific desirable trait of highly adapted and genetically balanced sugarcane cultivars, without involving a sexual cycle. The ability of maintaining the mutant genotype through vegetative propagation was also seen as an added advantage.

Spontaneous mutation involving easily distinguished character, such as colour of canes, is a common feature in sugarcane, especially in the cultivated species, *S. officinarum*. Barber (1906) isolated a naturally occurring sport called 'Gillman', which arose as a unicoloured sport from the clone Striped Mauritius. Thomas (1932) and Venkatraman (1935) isolated striped and coloured mutants in Co 213. Moir (1932) showed that, apart from colour of stalks, mutations may also occur in other characters of the plant. Some of the original canes (*S. officinarum*) are known to have a regular sporting cycle of light, striped and dark colour modifications. Striped forms of a number of cane varieties have arisen through mutations a few years after they are bred. It is unknown to what extent such somatic mutations take place in nature, for economically important characters such as yield, sugar, disease resistance etc. (Stevenson, 1965).

In considering the possibility of utilizing induced mutations for sugarcane improvement Price and Warner (1960) had postulated two approaches: firstly mutagenic treatment of cuttings and subsequent selection of mutants from vegetatively increased clones; secondly incorporation of mutation induction into existing breeding programmes so that, through segregation, recombination and selection in successive generations, unfavourable mutations might be rejected and favourable ones are accumulated. The breaking of linkages between desirable and undesirable genetic loci and evolution of novel recombinations were also envisaged.

S.M. Jain, D.S. Brar, B.S. Ahloowalia (eds.), Somaclonal Variation and Induced Mutations in Crop Improvement, pp. 421–446.

History

The first attempt to induce mutations in sugarcane was made in Hawaii, in 1927, when two sugarcane clones were treated with X-rays. No visible changes were observed in plants other than coloured stripes on rind (Heinz and Tew, 1987). Rao (1954) used X-rays to induce mutations in cultivated sugarcane varieties and isolated early-flowering mutants, and also obtained various shades of stripes on internodes. Tysdal (1956) exposed vegetative cuttings to neutron radiations and recorded morphological changes and retarded growth. In 1956, researchers at the Hawaiian Sugar Planters' Association Experimental Station exposed H 37-1933 cultivar to gamma-rays and selected a distinct morphological variant in yield to the original clone. Panje and Prasad (1960) isolated many plants with foliar abnormalities as a result of radiation-induced killing of cells and subsequent growth as different sectors. Similar observations were made by Vijayalakshmi and Rao (1960) in *Saccharum* species and hybrids, when the vegetative buds were exposed to different doses of gamma-rays. A mutant resistant to red rot from a highly susceptible variety Co 449 was isolated from gamma-ray-treated setts (Rao *et al.*, 1966). Hrishi and Marimuthammal (1968) obtained mutations for shortened internodes and glabrous leaf sheath in Co 419 using pyrogallol, pyrocatechol, quinol and acenaphthene. They reported that, for qualitative characters, the mutagenic effects were positive for number of internodes and brix percent, and negative for stalk diameter. Rao (1972) obtained non-flowering mutants. Mutants with glabrous leaf sheath from varieties with leaf sheath spines, *viz.* Co 527 and Co 419, were obtained by Jagathesan and Sreenivasan (1970). They also reported several mutants for colour changes and stripes on canes, and drastic mutants with thin canes in varieties Co 312, Co 419, Co 527 and Co 997. Walker and Sisodia (1969) isolated non-flowering mutants from gamma-irradiated material of commercial varieties B 49119 and B 52107. Nair (1973) reported a red rot-resistant mutant of Co 997 which was similar to the parent clone with no significant differences in yield, sucrose in juice or commercial cane sugar per hectare. Jagathesan and Ratnam (1978) succeeded in isolating a fast-growing vigorous mutant of Co 527 from plants obtained from gamma-ray-treated setts. Jagathesan (1982) reported several sugarcane smut disease-resistant mutants of Co 740 and Co 1287. Urata and Heinz (1972), Braux (1975), Siddiqui *et al.* (1976), Lo (1977) and others reported isolation of plants with changed characters such as herbicide resistance, changes in stalk size, sucrose percentage, etc. However, these efforts have not produced any commercially important variety.

Material

Vegetative buds which are the commercial planting material in sugarcane and 'true' seed used for evolution of new varieties through sexual breeding programmes were used as experimental material for irradition. In the case of axillary vegetative buds, the apical meristem appears as a smooth hemispheric dome tightly en-

closed by bud scales. It is reported (Thielke, 1960) that the sugarcane bud meristem has no tunica and corpus cellular organization typical of most of the angiosperms.

Studies on Radiosensitivity

Vijayalakshmi and Rao (1960) studied for the first time the radiosensitivity of *Saccharum* species. The upper limit of the safe gamma-ray dosage for normal growth of *Saccharum* species was found to be about 3000. Jalaja (1972) made the most comprehensive study on radiosensitivity of *Saccharum* spp. using X-rays, gamma-rays and chemical mutagens. The species studied included *S. officinarum*, *S. barberi*, *S. sinense*, *S. robustum* and *S. spontaneum*. The indices of radiosensitivity used were germination and survival, chromosome aberrations in root tips and pollen mother cells, and pollen fertility and nuclear volume.

The treatment conditions for all the clones studied were practically the same and the moisture content of the buds ranged from 58% to 83%. The clones included in this study were Mauritius 55 str, 57 NG 78, NC 104, Fiji 26 (*S. officinarum*), Lalri (*S. barberi*), Khakai, Chinia (*S. sinense*), 28 NG 219, 57 NG 11, 57 NG 208, NH 1 (*S. robustum*), SES 248, SES 106 B, Coimbatore, Thai 14#4, SES 91 and Imp 569 (*S. spontaneum*). A differential response of different genotypes of the same species to X-ray and gamma-ray 1–5 kr treatments were evident with respect to germination and survival. Clones NC 104 of *S. officinarum* and NH-1 of *S. robustum* were comparatively radioresistant and also showed an increase in germination and survival. The *S. spontaneum* study included clones with 2n = 40, 48, 64, 80, 90 and 112 chromosomes. No relationship between chromosome number and radiosensitivity could be observed in this species. *S. sinense*, clones Khakai and Chinia, showed a significant increase in germination and survival.

The response of these species in combination treatments involving X-rays and gamma-rays and 0.01% ethylmethanesulphonate as post-treatments increased germination and survival. These results indicated that there is a 'protection' (recovery from radiation damage) when irradiated and water-pre-soaked vegetative buds are treated with EMS.

A stimulatory effect of mutagens was recorded in seedling height when measured up to 35 days after the treatment in all clones of *S. officinarum*, *S. barberi* clone Lalri, *S. sinense* (Chinia and Khakai), *S. robsutum* 28 NG 219 and 57 NG 208 and *S. spontaneum* SES 248 and Coimbatore). However, this type of response was not uniform. Two types of stimulatory effects were noticed: the first was an increased germination of irradiated buds and the other was an increase in settling height.

Settling abnormalities recorded were chlorophyll variants and foliar abnormalities. An increase in abnormalities with the increase in dose of mutagens was recorded in some clones, whereas no such relationship was observed in others. No chlorophyll variants or foliar abnormalities occurred in the species *S. spontaneum*.

Cytological Studies

Chromosome aberrations such as breaks, minutes, shattered chromosomes, mosaics, laggards, etc. were recorded in the root-tip cells of *Saccharum* species treated with gamma-rays and combination treatments of gamma-rays and 0.1% EMS. An increase in the percentage of abnormal cells with increase in dosage of physical and chemical mutagen was recorded at the somatic mataphase. Combination treatments involving gamma-rays and EMS resulted in a higher percentage of abnormalities (Jalaja, 1972).

Meiotic abnormalities in gamma-irradiated plants of *Saccharum* included occurrence of many nucleoli, univalents, multivalents, bridges, fragments, minutes and laggards in all the species studied. Chemical mutagen treatments with diethyl sulphate and nitrosomethylurea also recorded abnormalities at a higher frequency compared to the controls. It is suggested that, by the time floral initiation takes place, abnormal cells, which are deleterious, might have been eliminated (Jalaja, 1972).

Based on the relative frequency of aberrations recorded in root tips, Jalaja (1972) indicated that *S. barberi* was a highly radiosensitive species with the highest number of aberrations, and *S. sinense* the most radioresistant species with the lowest number of aberrations. *S. officinarum* and *S. robustum* showed an almost similar response. Jalaja (1972) stated that there was no relationship between chromosome number and number of aberrations induced, since both *S. barberi* and *S. sinense* have a much higher chromosome number than the other two species, *S. officinarum* and *S. robustum* (2n = 80).

Pollen Radiosensitivity

The pollen of four clones of *S. robustum* and eight clones of *S. spontaneum* with somatic chromosome numbers ranging from 2n = 40 to 126 was used for irradiation with X-rays and ultraviolet rays. The LD_{50} doses for the clones studied are presented in Table 1. The data suggested that pollen of *S. spontaneum* and *S. robustum* were highly radioresistant. No relationship was observed between chromosome number, pollen volume, nuclear volume, chromosome volume and radiosensitivity (Jagathesan and Jalaja, 1969).

Induction of Mutations for Economically Important Characters

Srivastava *et al.*, (1989) used both physical and chemical mutagens for treating vegetative buds and found the LD_{50} level at 0.8% for EMS and 6 kr for gamma-rays. By repeated clonal selection mutants for various morphological characters were isolated. Screening for red rot disease resistance was done at vegetative mutation generations 2 and 3 (MV_2 and MV_3). An induced mutant combining red rot disease resistance with improved leaf characters was isolated from gamma-ray-

Table 1. LD_{50} of X-rays and UV rays of *Saccharum* species

Species/ clone	Somatic chromosome no.	Moisture content (%)	Pollen volume (μ^3)	Interphase nuclear volume (μ^3)	LD_{50} of UV rays (x 3 x 10^4 erg/cm^2	LD_{50} of X-rays
S. robustum						
Mol. 4503	60	32.58	28 606.53	350.91 ± 0.1743	126.5	224.9
57 NG 11	60	47.06	18 081.19	167.04 ± 0.1277	107.6	296.2
57 NG 208	80	51.02	29 659.73	486.98 ± 0.4949	212.6	331.7
28 NG 251	80	47.44	28 201.32	542.88 ± 0.5143	214.0	207.3
S. spontaneum						
SES 248	40	40.98	57 929.14	192.80 ± 0.1143	82.9	214.8
SES 106 B	48	38.39	60 128.75	392.48 ± 0.1816	55.5	241.0
SES 401	64	36.48	84 234.68	398.12 ± 0.1888	89.3	195.9
Coimbatore form	64	41.19	32 282.69	350.99 ± 0.1984	105.2	219.8
SES 515/3	80	38.33	34 642.28	453.66 ± 0.1431	178.4	234.1
SES 91	96	63.10	30 049.32	423.59 ± 0.1632	171.9	249.8
IMP 569	112	26.73	33 373.16	706.86 ± 0.1601	44.8	283.2
SES 559	126	50.78	29 152.85	772.51 ± 0.2326	202.6	303.2

treated susceptible variety CoJ 64. The lower dose of 4 kr had a higher frequency of resistant clones. These results indicated that gamma-ray treatment was superior to chemical mutagens for inducing mutations in sugarcane. Srivastava *et al.*, (1986), working with chemical mutagens such as ethylmethanesulphonate, diethylsulphate and nitrosomethyl urea, obtained no red rot-resistant mutant. The chimaera resulting from multicellular bud structure appeared to be the major handicap in obtaining consistent results in the MV_1 generation.

Due to the relatively large size of the bud meristem, the occurrence of various types of chimaeras and diplontic or intrasomatic selection is a common feature in sugarcane. Various methodologies have been tried to increase the spectrum of mutations and to overcome diplontic or intrasomatic selection (Bhagyalakshmi, 1976). Growing of mutagen-treated vegetative buds at a higher level of nitrogen, decapitation of MV_1 plants at different intervals and closer planting of MV_1 generation in three popular varieties, Co 312, Co 419 and Co 453, were attempted. A marked increase in the percentage of deviant lines in nitrogen doses of 100 and 300 kg was noticed in the MV_1 generation of Co 775. These two nitrogen level treatments also recorded an increased frequency of colour changes. Decapitation of plants carried out on the 90th and 105th days after irradiation increased the frequency of changes. It is possible that the initials of tillers, already present at the time of irradiation, developed into tillers due to the decapitation of the mother shoot, resulting in a higher frequency of mutations. The closer planting of MV_1 plants restricted the number of tillers and hence the mutation frequency increased (Bhagyalakshmi, 1976). A varietal difference was noticed.

Jagathesan (1976) summarized the mutation breeding work carrried out at the Sugarcane Breeding Institute. The list of varieties treated with mutagens, and the type of mutations obtained, are presented in Table 2.

Jagathesan (1976) particularly mentioned two mutants, M-10 and M-13, obtained from Co 527. The M-10 mutant was early-maturing and can be harvested after $8^1/_2$ months (Jagathesan and Ratnam, 1978). The second mutant, M-13, from

Table 2. Effect of different mutagens on inducing mutations in several sugarcane varieties

Sugarcane variety	Type of mutagen used	Nature of mutation obtained
Co 419	Gamma-rays 3, 5 and 7 kr	Earliness
Co 527	Gamma-rays 3 and 5 kr	Glabrous leaf sheath. Non-flowering, early maturity
Co 1287	Gamma-rays 3, 5 and 7 kr; EMS 0.10–0.80%	Smut resistance; increase in sucrose content
Co 740	EMS 0.10–0.80%	Smut resistance
Co 775	Gamma-rays 3, 5 and 7 kr	Glabrous leaf sheath, red rot resistance.

Data from Jagathesan (1976).

the same variety, is non-flowering and yields better than the donor (Jagathesan, 1979). However, these mutants were not identified for commercial cultivation. Reports on the identification of red rot disease-resistant mutants are available. Rao *et al.*, (1966) isolated a red rot-resistant mutant from Co 449 cultivar, and Nair (1973) reported another red rot-resistant mutant from Co 997 cultivar. These mutants were also not planted commercially.

Balasundaram (1981) studied cane yield and sucrose content of induced mutants in sugarcane, which are controlled by polygenes. Several mutants, especially drastic mutants, had a higher percentage of sucrose than the donor variety Co 419. High sucrose type mutants occurred in a much higher frequency than the high-yielding type of mutants. Two mutants, 368 and 419/1, had higher cane yield and higher commercial cane sugar than the parent Co 419 cultivar. Drastic mutants of Co 419, isolated after 7.5 kr X-ray treatment, had 2n = 80–85 chromosomes when compared with normal chromosome number 2n = 110–115 for Co 419, indicating that in the origin of these mutants extensive chromosome losses have taken place. After 4 years of vegetative propagation, no segregation had occurred, indicating stability (Jagathesan and Jebadhas, 1976).

Sugarcane smut disease-resistant mutants were isolated by gamma-ray treatment of CoC 671. The irradiated setts were planted and screened from MV_2 onwards to MV_6. A yield evaluation trial of 10 mutants resulted in identifying three mutants. They had characteristics similar to the parental cultivar CoC 671 in terms of yield and quality, and with the additional advantage of smut resistance (Radhakrishnan, 1990).

Chemical Mutagenesis

Hrishi and Marimuthamal (1968) used five chemical mutagens, namely pyrogallol, pyrocatechol, quinol, acenaphthene and coumarin to induce mutations in sugarcane. The optimum strength for induction of mutations and tissue damage appeared to be between 0.01 M and 0.001 M for pyrogallol, pyrocatechol and quinol. Mutations for shortened internodes and glabrous leaf sheath were induced by pyrogallol and pyrocatechol. Tissue damage such as partially dissected lamina occurred when pyrogallol, pyrocatechol and acenaphthene treatments were applied. Pyrocatechol and acenaphthene induced profuse tillering, whereas quinol and coumarin suppressed tiller formation. Hrishi *et al.*, (1968) reported induced variability through pyrogallol and pyrocatechol treatment for a number of internodes, stalk diameter and brix percentage. Similar to the induced mutants with physical mutagens, commercially viable selections were not possible from these studies either.

In a study combining chronic irradiation and callus culture, Nagatomi (1993) reported an increase in mutation frequency rate. The plant population regenerated from young leaf tissue of chronic irradiated plants, grown under a gamma-field receiving a total dose of 300 and 100 Gy, showed a wider variation in qualitative characters than control plants. This variation extended in both negative and

positive directions. Variation and heritability, in the broad sense, of most of the agronomic characters increased significantly among the sub-clones as the irradiation dose increased. The method, combined with chronic irradiation and tissue culture, is evaluated as an effective method of widening the mutation spectrum and increasing the mutation frequency in regenerated plants.

True Seed

Only two centres, the Hawaiian Sugar Planters Association Experiment Station, Hawaii, USA and the Sugarcane Breeding Institute, Coimbatore, India, explored the possibility of using true seed for the induction of mutations. At Hawaii, true seeds from eight sugarcane clones were irradiated to induce translocations. Dosages beyond 16 kr were detrimental for seedling survival. Cytological studies did not indicate any increased frequency of multivalents at meiosis as a result of irradiation (Heinz, 1973).

At the Sugarcane Breeding Institute, Coimbatore, India, true seeds from seven inter-varietal crosses were irradiated with 5, 10 and 15 kr gamma-rays (Jebadhas, 1982). The seedlings were screened in a similar way to the sexual breeding programme without making any specific effort to identify plants with chromosomal aberrations. After the normal selection in four clonal generations, one clone was identified as a promising variety, Co 8153. There was no way of assessing the role of irradiation in the evolution of this clone.

Information accumulated over the years on mutation breeding in sugarcane indicates that genetic variability could be created in sugarcane through induced mutations. Among the mutagenic agents, gamma-irradiation was more potent than X-rays or chemical mutagens. Most of the mutations documented relate to morphological changes, which are easily detectable. These morphological characters are probably under the control of a few major genes and any changes in them result in the mutated character. The complexity of the genetic make-up of sugarcane and most of the desirable traits, such as improvement in disease resistance, yield or quality, are under polygenic control. It is very difficult to improve further through mutation breeding. Due to the vast untapped genetic variability available in the world collection of sugarcane germplasm, there is no compelling reason for mutation breeding in sugarcane. Interest in mutation breeding has almost ceased in most of the sugarcane-growing countries due to limited success.

Chimaerism in mutagen-treated populations throws doubt on the repeatability of the mutants over vegetative generations. Therefore, it was essential to grow mutants up to four or five vegetative generations. This lessened the advantage of identifying a new variety in a shorter time. By the time a mutant with a desirable trait in a variety is produced through mutation breeding the conventional system is able to evolve new varieties with improvement in many desirable characters. Although, some elite clones were improved through induced mutagenesis, e.g. non-flowering, resistance to diseases such as smut, red rot, etc., none of these varieties is yet planted in large areas. However, with the development of methodolo-

gies such as plant cell, tissue and organ culture, and the ease with which a mutation breeding programme can be superimposed on a tissue/cell culture system, induction of mutations, especially for inducing chromosomal aberrations for repatterning the *Saccharum* genome, may become important in the future. Refinement of techniques to detect even minor modifications through molecular means will certainly help to utilize mutational events in future programmes.

Somaclonal Variation

Sugarcane tissue culture work was initiated by the Hawaiian Sugar Planters Association Experiment Station, Hawaii, USA (HSPA) in 1961 (Nickell, 1964, 1967). The cytogenetic studies on the subclones produced through the differentiation of the stem paranchyma callus of H 50–7209 demonstrated variation in chromosome number for each of the five different subclones. The morphological variation observed was thus due to the variation in chromosome number. Larkin and Scowcroft (1981) introduced the terms 'somaclones' for plants derived from any form of cell culture, and 'somaclonal variation' for the variation displayed among such plants. They cited examples of extensive somaclonal variation from sugarcane tissue culture.

The variability occurring in sugarcane callus cultures was reported early by researchers from the HSPA (Heinz and Mee, 1969, 1971; Heinz, 1973; Heinz *et al.*, 1977). The initial studies on variability among 'somaclones' were made on plants differentiated from two varieties, H 37–1933 and H 50–7209, which included variation in chromosome number, four-enzyme systems and general morphology of the plants (Heinz and Mee, 1971). All eight plants of H 37–1933 had chromosome number 2n = 106, similar to the donor. However, H 50–7209, which is a known chromosomal mosaic (Heinz *et al.*, 1969), when cultured, produced somaclones having 2n = 94 to 2n = 120 chromosomes. One somaclone was exceptional, with the chromosome number ranging from 2n = 17 to 118 (Heinz and Mee, 1971). They had indicated that part of the chromosomal variation was due to the asynchronous division of multinucleate cells. The frequency of plants with wider variation in chromosome number occurred in *in-vitro* cultures rather than in intact plants. This phenomenon is probably due to a better chance for survival of highly variant cells *in vitro* than *in vivo*. Somaclones with such variation in chromosome number differ in phenotypic expression, ranging from normal to dwarf plants lacking vigour. The plants are stable, and show no chimaerism.

Somaclonal Variation for Economically Important Characters

The Hawaiian work clearly demonstrated that the variability noticed in sugarcane somaclones had a genetic basis. This system is inducted as an adjunct to conventional breeding for sugarcane improvement programmes in a few countries, for rectifying one or two specific defects of sugarcane cultivars.

Fiji and Downy Mildew Diseases

Krishnamurthi (1974), Krishnamurthi and Tlaskal (1974) produced somaclones from a large number of sugarcane varieties and screened for their reaction to Fiji disease (virus) and downy mildew (*Sclerospora sacchari*). Somaclones exhibited an increased resistance to both the diseases. Somaclones of cultivar pindar, (resistant to Fiji and downy mildew) were tested for cane yield and quality and were found to be similar to the donor. On the basis of these results, a cooperative programme between Hawaii and Fiji was undertaken to screen somaclones. A high frequency of resistant plants was obtained (Heinz, 1976).

Helminthosporium Eye Spot

Heinz *et al.*, (1977) observed variability for eye spot disease caused by *Helminthosporium sacchari*. Many resistant clones were isolated without showing any visual differences from the parent. Incorporation of chemical mutagens, gamma-ray treatments and *in-vitro* selection were superimposed to increase the spectrum, frequency and efficiency of selection. However, the eye spot-resistant somaclones of two Hawaiian varieties which initially showed resistance better than the parent, lost their resistance after 10 years of vegetative propagation (Maretzki, 1987). The origin of a new physiological race of the pathogen might also be responsible for the susceptible reaction.

Larkin and Scowcroft (1983), working with sugarcane cultivar Q 101, which is an agronomically valuable Australian cultivar but susceptible to eye spot, produced somaclones and screened plants using a leaf assay technique for quantifying the sensitivity of the leaves to *Helminthosporium sacchari* toxin. A high percentage (8.9%) of somaclones were highly resistant. These resistant somaclones maintained resistance in successive vegetative generations. Lat and Lantin (1976) studied eight somaclones of cultivar CAC-57–13 and observed significant differences in stalk diameter, length and weight as compared to the parental cultivar.

Smut

Cultivar CoC 671 is high-yielding with high sugar content and is extensively cultivated in tropical India. It is susceptible to sugarcane smut disease. Overall 94 somaclones were screened for smut disease. Somaclones showing resistance comprised 67%; 8.5% were moderately resistant (MR), 19.5% moderately susceptible (MS) and 5.3% susceptible (S), like the donor parent. Agronomically superior or similar clones to the donor were selected for further multilocation evaluation. Following this study, selections from the sexual breeding programme, which are rejected at the final stages due to smut susceptibility, are routinely passed through a tissue culture cycle. Results of these studies conducted over the years are presented in Table 3.

Table 3. Data on screening of somaclones for smut disease resistance

Variety	No. tested	R (%)	MR (%)	MS (%)	S (%)	HS (%)
L 78–420	153	36 (23.53)	30 (19.61)	40 (26.14)	24 (15.67)	23 (15.03)
Co 7704*	158	87 (55.06)	33 (20.89)	27 (17.09)	6 (3.80)	5 (3.16)
Co 740	150	–	–	–	–	150 (100%)
Co 85–261	120	8 (6.67)	7 (5.83)	21 (17.50)	19 (15.83)	65 (54.17)
Co 6907	201	18 (8.96)	4 (1.99)	30 (14.93)	34 (16.93)	115 (57.21)
G 86–20	226	62 (27.43)	32 (14.16)	50 (22.16)	39 (17.26)	43 (19.03)
CoC 671	94	63 (67.02)	8 (8.51)	18 (19.15)	5 (5.32)	–

R = Resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, HS = highly susceptible.
*Moderately resistant to smut.

The donor parents of the somaclones tested here are hybrids, synthesised from three or four species of *Saccharum*. They are all susceptible to sugarcane smut disease. In most of the clones studied, there is a shift towards 'resistance', except in Co 740, where R or MR types could not be obtained. The recovery of all classes of susceptibility MR, MS, S and HS types shows that it is possible to obtain genetic variability for smut disease resistance after reorganization of genome through a callus culture cycle (Jalaja *et al.*, 1987), which is either chromosomal rearrangements or aneuploid chromosome variation. In hybrid crop species (Armstrong *et al.*, 1983; Ahloowalia, 1983), most of the *Saccharum* species and hybrids are chromosomal mosaics (Nair, 1972; Sreenivasan and Jagathesan, 1975), and extensive somaclonal variation in callus-derived plants is attributed, in part, to pre-existing mosaicism (Heinz *et al.*, 1977; Krishnamurthi and Tlaskal, 1974: Sreenivasan and Jalaja, 1982a,b; Sreenivasan and Sreenivasan, 1984). However, there is evidence indicating that the variation occurs during the tissue culture cycle (Scowcroft, 1984). In sugarcane both chromosomal numerical and structural aberrations seem to occur extensively as a result of doubling of the chromosomes at various levels during interspecific hybridization; these repatternings are well tolerated.

Cytological studies were made on three cultivars, CoC 671, Co 740 and Co 7704. Extensive morphological variation among somaclones was observed only in Co 7704. Cultivar Co 740 did not show any variation in morphology or smut disease resistance. In CoC 671 somaclones, morphological variation was confined to cane colour variations and waxiness on the canes. Cytologically, Co 7704 had the widest variation in chromosome number, which ranged from 2n = 100 to 2n = 121 (Sreenivasan and Jalaja, 1981). Variation in chromosome number was limited in the other two clones. The same situation was also seen in other sugarcane cultivars (Heinz *et al.*, 1977). Chromosomal variation is not a prerequisite for somaclonal variation; however, it enhances the frequency of variants. The

methodology could be effectively utilized to improve smut disease resistance in sugarcane cultivars, without adversely affecting other desirable traits.

Rust

Sugarcane rust disease is caused by *Puccinia melanocephala* H and P Syd. (*P. erianthi* Padw. et A. Khan). In susceptible varieties, the fungus causes extensive damage to the leaves, leading to their drying. Even young and photosynthetically active leaves are infected, causing yield losses. Cultivar CoA 7601, an early-maturing high-sugar variety, had to be withdrawn from cultivation due to the emergence of rust. A total of 261 somaclones were produced and field-planted along with the donor parents. The results of the screening are given in Table 4.

Rust-resistant somaclones morphologically resembled the donor parent to a large extent, and retained the earliness and high sucrose content. In order to assess the stability of resistance, explants from the resistant somaclone were induced for callusing and plants were regenerated. None of the 308 plants established in the field showed any symptom of rust, indicating maintenance of stability of resistance in somaclones. In a vegetatively propagated heterozygous complex hybrid such as sugarcane, this method could be used to assess the stability of the genetic variation, because sexual breeding methods will not produce the desired information on the segregation pattern (Sreenivasan *et al.*, 1987). This somaclone was identified as Co 92007 after multilocation evaluation.

Red Rot

CoC 671 and CoJ 64 are two Indian commercial sugarcane cultivars with early maturity and high sucrose. These two varieties are susceptible to red rot disease. So far it has not been possible to identify any resistant somaclones (Jalaja and Sreenivasan, 1990a). By incorporating a semipurified toxin from red rot fungus, somaclones were produced from Co 7717. There are indications of an enhanced resistance reaction. From about 700 somaclones screened, 120 showed moderately resistant (MR) reaction under laboratory test conditions (Mohanraj, 1996). Irradiation of the callus, subsequent plant differentiation and screening also did

Table 4. Rating of somaclone for sugarcane rust disease.

0	1	2	3	4	5	6	7	8	9	Total plants screened
1	–	–	–	–	5	–	14	16	225	261

Number of plants showing susceptibility on a 0–9 scale0 = Resistant, 9 = susceptible.

not yield any resistant clones in two varieties, Co 8334 and CoJ 64 (Jalaja and Sreenivasan, 1990a).

Sugarcane Mosaic

Nickell and Heinz (1973) reported having isolated sugarcane mosaic disease-resistant somaclones from susceptible sugarcane varieties. Recently, Oropeza *et al.*, (1995) have isolated somaclones resistant to sugarcane mosaic polyvirus (SCMV) from a susceptible variety PR 62258 via somatic embryogenesis by increasing the number of subcultures of embryogenic cultures. Resistant somaclones AT 626 and BT 627 maintained the resistant trait over 7 years of field testing.

From two sugarcane mosaic virus-susceptible varieties, Co 419 and 54 C9, Fahmy (1990) identified somaclones resistant to SCMV strains A, B, D, H and I. Other somaclones exhibited various degrees of resistance and susceptibility. Resistant, tolerant and the least susceptible somaclones had higher values compared with the control with respect to plant height, stalk diameter, stalk weight and total soluble solids.

Gumming Disease

In an attempt to isolate somaclones resistant to gumming disease (*Xanthomonas campestris* pr. *vasculosum*). Bonnel *et al.*, (1988) produced plants from cultivar R 472 and screened somaclones under natural and artificial infection conditions. No resistant variants could be identified. However, major changes were observed in a somaclonal population, such as increased tillering, reduced stalk weight and reduced flowering rate. In the case of *Puccinia melanocephala*, which causes sugarcane rust disease, susceptible somaclones could be isolated from resistant variety H 70-0144 and resistant somaclones from susceptible clone H 74-0922.

Other Characters

Liu and Chen (1976) selected 417 somaclones from eight sugarcane varieties among the 4600 somaclones generated from the callus of shoot apices. The callus derivatives of the eight cultivars varied in morphological traits from their donors, ranging from 1.8% to 34.0%. Auricle length showed the highest (86%), difference dewlap shape (6.5%), hair groups (6.2%), and attitude of the top leaf (1.9%). The sucrose percentage of the somaclones increased over the donor from 2% to 12%. Chromosome number varied from 2n = 86 to 176 in the F 156 derivatives when compared with donors, 2n = 114; range of variation 2n = 88–128 was seen in F 164 as compared with the control chromosome number 2n = 108. The somaclones of F 146 generally centred around the original chromosome number, 2n = 110, and it was the most genetically stable cultivar.

A salt-tolerant cell line was selected from cell cultures of F 164 in the presence of 1.5% NaCl. A somaclone 70-6132 of F 164 had 32% higher cane yield, 34% higher sugar yield and 6% more stalks than its donor in the field tests. Clone 74-3216 of F 160, one of the major commercial varieties of Taiwan, improved by 5%, 2% and 14%, respectively for these traits over its donor. Another somaclone, 75-3070, was 80% and 15% better for cane yield and sugar yield, respectively. A somaclone, 71-4829, isolated from H 37-1933, was significantly superior to the donor in sucrose content. Somaclone 76-5530 of F 177, a sugarcane smut disease-susceptible cultivar, was found to be more resistant than the donor parent (Liu and Chen, 1982). Liu and Yeh (1982) isolated NaCl-tolerant somaclones from cultivar F 177.

Sugarcane variety Co 7704 is commercially grown by few sugar producers in India. This variety is resistant to smut and red rot disease and has good yield and juice quality. It suffers from a specific defect, drying of 3rd and 4th leaf from the spindle, between 90 to 120 days during summer. Somaclones produced from leaf callus and plants without leaf drying were selected. Out of 225 somaclones grown to maturity, 185 clones showed no drying of leaves. After yield and quality evaluation, 14 clones were finally selected (Sreenivasan and Jalaja, 1983). One of them, Co 85007, has performed well in some locations while evaluating in multilocation trials. This clone was tolerant to salinity. The variety showed no leaf drying even after 15 years of vegetative propagation.

Leaf sheath spines is an undesirable character in sugarcane, especially in countries where harvesting is done manually and sugarcane tops are used as cattle feed. Cultivar Co 7717 is high-yielding with high sugar content and is popular in some parts of subtropical India. Somaclones produced from Co 7717 showed no variation for this character. Subsequently callus was subjected to 3 and 4 kr gamma-irradiation and plants were differentiated from the irradiated callus. Out of 207 somaclones screened, eight plants had glabrous leaf sheath. Clone Co 91017 was selected after multilocation trials (Sreenivasan and Sreenivasan, 1994). It is thus obvious that, when desired variation is not obtained through callus induction and plant differentiation, variation can be induced by using physical mutagens to increase the spectrum of variation.

Cellular Selection

Cellular selection by applying appropriate selection pressure to the undifferentiated cell population has also been in operation for sugarcane improvement. Maretzki (1987) has indicated several types of selection for cells which can survive under the chosen regime.

1. Cells that pre-existed in the tissue, and proliferated prior to application of selection pressure, were favourable to the *in-vitro* conditions.
2. These cells originated from the same tissue, however, remained as a minor component of the total *in-vitro* cell population until the selective agent was applied.

3. Somaclones produced under *in-vitro* culture conditions.
4. Mutants triggered by the selection agent itself.

The success of the system is determined by: (a) the ability of the cells to differentiate into plants, (b) the expression of the character selected in the whole regenerated plant, and (c) the stability of the phenotype through vegetative propagation.

In sugarcane, variants with higher tolerance to *Helminthosporium sacchari* (Heinz *et al.*, 1977; Larkin and Scowcroft, 1983), and smut disease caused by *Ustilago scitamineae* (Peros and Chagvardieff, 1983) were reported. However, the methodology is not extensively used. The cellular selection procedure similar to the selection for salt tolerance was also used by many researchers (Fitch and Moore, 1981; Liu and Yeh, 1982; Jalaja and Sreenivasan, 1995; Naik and Babu, 1988). Some of the plants regenerated from salt-tolerant cell lines are reported to have remained stable over vegetative generations (Sanjiva Reddy, 1994).

Interspecific and Intergeneric Hybrids

Besides causing alterations in chromosome number, tissue culture can alter the structure of individual chromosomes. Cryptic modifications of the genome include chromosome breakage reunion events, reciprocal and non-homologous translocations, acentric and centric fragments and repositioning of transposable elements which can modify the expression of neighbouring genes (Larkin and Scowcroft, 1981; Chourey and Kemble, 1982; Green *et al.*, 1977; Novak, 1980; Nakamura *et al.*, 1981; McCoy *et al.*, 1982). The resulting changes in genetic potential are likely to be more useful than aneuploidy or polyploidy for further crop improvement programmes, especially in interspecific and intergeneric hybrids of *Saccharum* where there is a restricted interspecific and intergeneric chromosome pairing.

Saccharum × Zea

The *Saccharum* × Zea hybrid was produced from a cross made in 1938 between *S. officinarum* clone Vellai and *Zea mays* var. Golden beauty with two B chromosomes (Janaki Ammal, 1941). This hybrid had 2n = 52 chromosomes, 40 from *S. officinarum*, 10 A chromosomes and two B chromosomes from maize. Callus initiation and plantlet differentiation were obtained from expanding leaf explants. Cytological studies of the callus and 12 subclones indicated numerical chromosome mosaicism in the callus and plant-to-plant chromosome number variation in the subclones. The variation in chromosome number was from 2n = 46 to 2n = 106 in callus cells and 2n = 48 to 2n = 56 in somaclones. These variations were shown to be, in part, derived from the parent plant. Marked morphological differences were noticed in leaf width and colour, as well as in vigour. Although, it was not possible to isolate tetraploids, one somaclone flowered normally, which was sterile (Sreenivasan and Jalaja, 1982a).

Saccharum × *Sclerostachya*

Sclerostachya fusca is a tall grass and grows well in waterlogged areas of sub-Himalayan terrain. As a member of the '*Saccharum* complex' (Mukherjee, 1954), it easily hybridizes with sugarcane. Intergeneric hybrids have been made to introduce waterlogging resistance, heavy tillering and earliness in sugarcane. One hybrid from the cross of *Saccharum officinarum* 2n = 80 and *Sclerostachya fusca* 2n = 30 was cultured (Sreenivasan and Sreenivasan, 1984). The hybrid had 2n = 55 chromosomes, 40 from *S. officinarum* and 15 from *Sclerostachya*. A total of 32 somaclones ultimately survived and 20 were used for the cytological studies. All plants had the chromosome number of the original hybrid, but one plant had a dicentric chromosome in 60% of cells and an acentric fragment in 10% of cells, showing that segmental exchange between these genera are possible. Morphological variations, observed in tillering, stalk diameter, erectness of leaves, leaf angle, stem epidermal pattern and in sugar yield, are caused partly by cryptic chromosomal rearrangements. This study indicated that cryptic structural changes are sufficient to cause somaclonal variation, and aneuploid chromosome variation is not necessary for such changes. Morphological variation among the somaclones of interspecific and intergeneric hybrids associated with chromosome structural variation has been reported in *Lolium* (Ahloowalia, 1976, 1978; Kasperbauer *et al.*, 1979), *Hordeum* (Orton, 1980; Orton and Steidl, 1980), *Triticum* (Mohmand, 1991; Li and Dong, 1994) and in many other instances.

Amuthavalli (1985) used the same hybrid for inducing amphidiploids by adding colchicine and kinetin in the culture medium. A total of 110 plants were screened for morphological changes in different treatments. Morphological differences were noticed with respect to canopy structure, leaf colour and number of tillers. An unselected population of 60 plants was cytologically studied. Twenty-one plants had either double or near-double somatic chromosome number 2n = 110;

Table 5. Frequency of somaclones derived from leaf callus of *Saccharum* × *Sclerostachya* hybrid with normal and doubled chromosome number

Treatment	Total plants screened	Diploid	Percentage of diploid plants	No. of amphidiploids	Percentage
	21	15	71.4	6	28.6
	17	6	35.3	11	64.7
	16	12	75.0	4	25.0
	6	6	100.0	0	0
Total	60	39	65.0	21	35.0

+Col = Callus grown on solid differentiating medium with 10 mg/L colchicine.

10 mg/L k = Callus grown on solid differentiation medium with 10 mg/L kinetin.

+Col 4D = Callus grown in suspension culture with 10 mg/L colchicine.

–Col 4D = Untreated callus in suspension culture.

39 plants had chromosome numbers around 2n = 55, similar to the donor parent. Thirty-five per cent of the somaclones studied were amphidiploids. However, the frequency of plants with such double chromosome numbers varied considerably in different treatments. Only 15% of plants showed constant 2n = 55 chromosomes in root tip cells. Among the plants with doubled chromosome number, chromosome number varied in different cells of the same plant.

The chromosome number varied from 2n = 102 to 120 in amphidiploids and from 2n = 47 to 69 in different somaclones. In addition to these euploids, aneuploid chromosome variation was also seen. A low frequency of plants showed structural chromosome aberrations, such as the occurrence of dicentric chromosomes and fragments at metaphase, laggards, and bridges at anaphase in root-tip mitosis. Some of these somaclones with chromosome translocations at meiosis (Figs 1 and 2) were used as parents into the regular breeding programme.

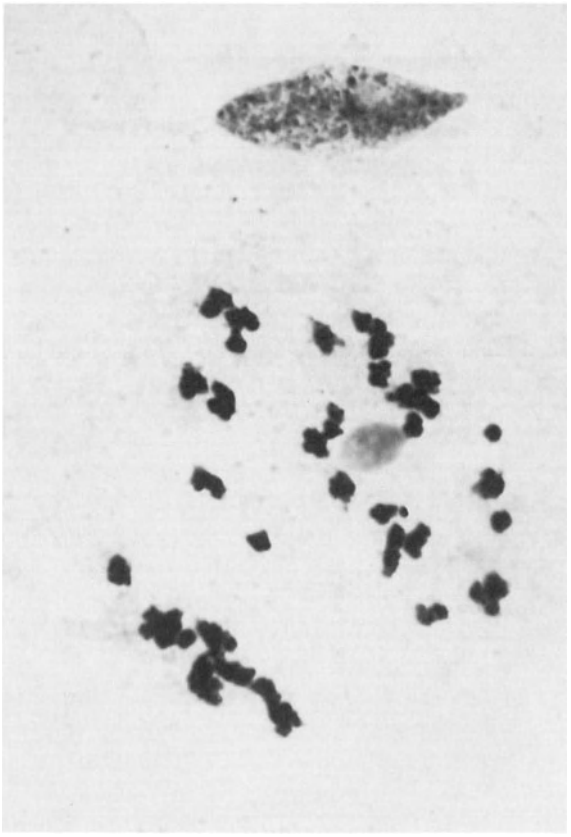

Figure 1. Cytology of a somaclone of *Saccharum* × *Sclerostachya* showing pentavalents, quadrivalents and trivalents

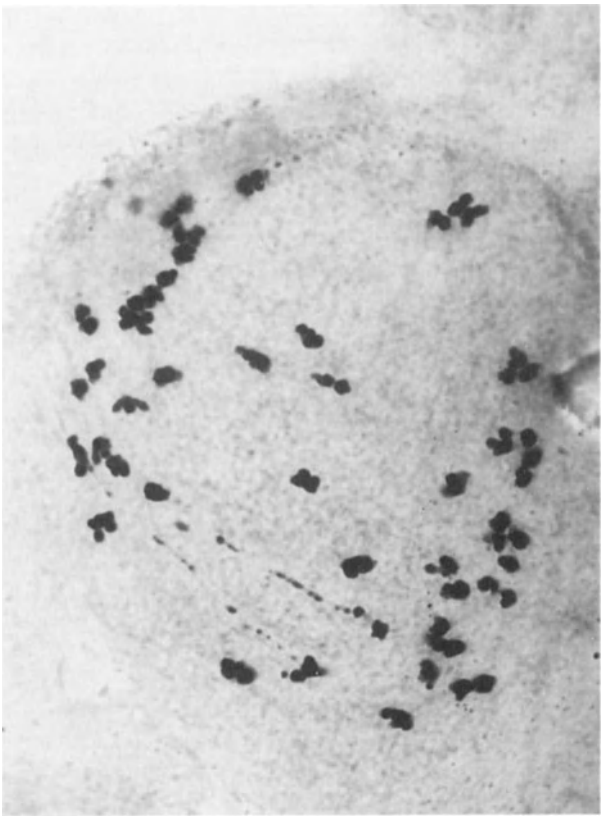

Figure 2. Meiotic anaphase showing bridges: *Saccharum* × *Sclerostachya* somaclone

Sugarcane × *Erianthus*

Nagai *et al.*, (1991) produced over 1600 somaclones from regenerating callus cultures of intergeneric hybrid clone H 83-9998 (2n = ca 94), involving *Saccharum* spp. hybrid H 62-4671 (2n = 115) × *Erianthus arundinaceum* (2n = 60) clone 28 NG 7. One hundred and twenty-six somaclones were selected for detailed study, based on observed morphological variation which included stalk number, stalk length, stalk diameter, leaf width and sucrose percentage. Variation in dewlap shape, leaf and stalk colour, leaf angle, rhizome habit and pollen fertility and flowering dates were obvious. Cytological studies and isozyme characterization confirmed the genetic basis of these changes. Chromosome number ranged from 2n = 66 to 2n = 109. With isozyme analysis, one to four band variations in three systems (peroxidase, phosphoglucomutase and phosphoglucoisomerase) were found. Significant genetic variance estimates and genetic CVs (6.7–25.6%) indicated that the differences observed among somaclones would probably remain stable over asexual propagation cycles. A few

variants showed directional resemblance to either *Saccharum* or *Erianthus* parent in morphology, chromosome numbers and isozyme pattern, clearly indicating that genetic variation, which probably cannot be achieved through sexual cycles, can be isolated through this pathway in intergeneric hybrids of sugarcane. Nine of the somaclones had higher estimated yield than the hybrid. This suggested that, with tissue culture, superior somaclones in agronomic performance might be obtained. Because intergeneric hybrids are sterile or partially fertile, back-crossing is either difficult or impossible to accomplish. Nagai *et al.*, (1991) isolated a few fertile types among the somaclones which will aid further back-cross programmes.

A hybrid involving *S. officinarum* clone Manjri Red × *Erianthus ciliares* was produced and cytologically screened (Rao *et al.*, 1963). The hybrid had 2n = 60 chromosomes. Somaclones were produced from leaf explants of this hybrid (Sreenivasan and Jalaja, 1984). Cytomorphological studies of these somaclones indicated the following:

1. Somaclones showed morphological variation with respect to number of root eye rows on the node, bud size, bud shape and extension, piping of the stem, leaf length and width, and time of flowering. A shift in flowering time of most of the somaclones towards *S. officinarum* parent was noticed.
2. All somaclones were male sterile like the donor. The pollen fertility of somaclones ranged from 1.51% to 6.08%.
3. Chromosome number of somaclones ranged from 2n = 50 to 60, with all the number represented in between.
4. Variation in chromosome number did not follow a specific pattern. Somaclone showed a higher frequency of 2n = 50 and 2n = 55 chromosome numbers.
5. Meiotic analysis of somaclones indicated deletions and duplications of different chromosomes.

Sugarcane × Sorghum

A similar study was made with a commercial hybrid involving sugarcane hybrid variety Co C 671 with a sweet sorghum variety Romo. The hybrid was sterile and had 2n = 57–60 chromosomes. Somaclones were produced and screened initially to isolate plants without precocious bud sprouting. Somaclones exhibited extensive chromosomal variation, accompanied by morphological differences. One somaclone, out of the several clones used as a female parent in crosses, yielded a few hybrid seedlings Cy 80–1 × Co 775. It was, thus, possible to use this sterile intergeneric hybrid for further back-crossing (Sreenivasan and Jalaja, 1987).

Cytological screening of 87 somaclones was performed. Out of these, 47 had 2n = 60 chromosomes. Thirty-one somaclones were of mixoploid nature. The chromosome number ranged from 2n = 55 to 64. Two plants had 2n = 60 + 1 fragment. Six plants had 2n = 62 and one each had 2n = 64, and 2n = 58 chromosomes. Stalk number, hand refractometer brix percentage (total solids in juice), and stalk diameter of 205 somaclones were studied. The range for brix percentage

was from 11.8% to 18.5%, stalk diameter from 2.1 to 2.7 cm and number of millable canes (NMC) one to 17.

In a complex sugarcane spp. hybrid Co 7201, root zone regions from immature nodes still enclosed by leaf sheath were cultured (Sreenivasan and Jalaja, 1985). Among the somaclones produced were three slow-growing plants. Cytological studies of these plants revealed that two had doubled chromosome number 2n = ca 220 and one had mixoploid number (Fig. 3). The mixoploid was used to produce a second cycle of somaclones. A total of 141 somaclones were planted in the field. Great variation occurred in morphology as well as in growth. Some were very dwarf, producing few short internodes. Chromosome number in different plants ranged from 2n = 200 to 220, which is in the tetraploid range. Chromosome number 2n = 132–136 is almost in the range of the donor.

Saccharum × *Miscanthus*

Saccharum × *Miscanthus* hybrids were extensively produced by the Taiwan Sugar Research Institute, Taiwan, especially for transferring disease resistance.

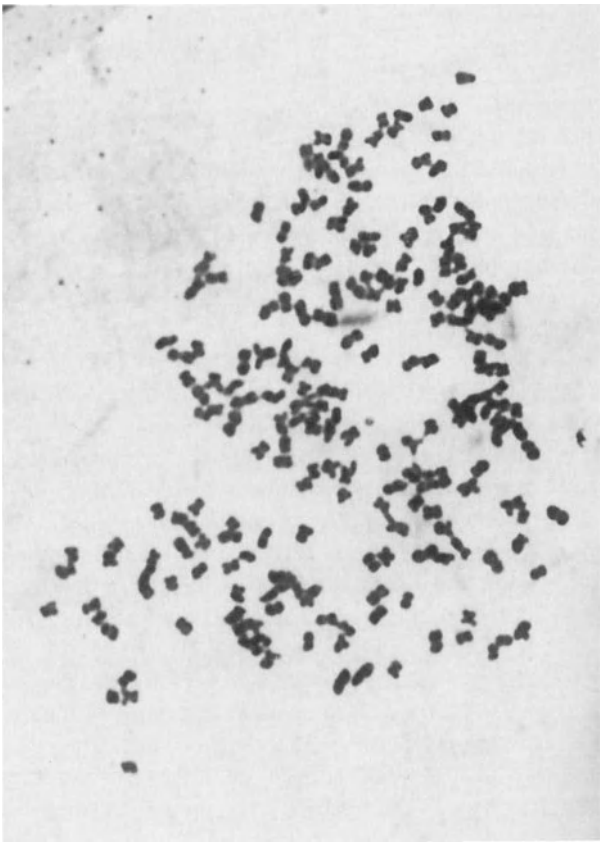

Figure 3. A tetraploid somaclone of sugarcane clone Co 7201

Colchicine-induced amphiploids from cell culture of *Saccharum* × *Miscanthus* hybrids were isolated (Chen *et al.*, 1992). Cytological examination of the somaclones showed a wide range of distribution of chromosome number from 2n = ca 103 to 220, separating them into two groups.

S. officinarum × *S. spontaneum*

Sreenivasan and Jalaja (1984) produced somaclones of interspecific hybrids involving *S. officinarum* × *S. spontaneum* (2n = 120 × 2n = 80). Two hybrids $^{2}/_{11}$ and $^{13}/_{85}$ with 2n = 100 chromosomes were cultured, and over 600 somaclones were established. They were screened for number of millable canes, stalk diameter, brix percentage and other morphological features. Variation for brix percentage was from 5% to 18%, for NMC/clump 2–37, stalk diameter 1.1–2.0 cm. A few somaclones resembled the *S. spontaneum* parent. The study revealed that it is possible to create novel genetic variability and select somaclones combining higher stalk diameter, higher stalk number and better brix, and also possible to avoid one back-crossing during further nobilization. The cytological studies clearly showed that substantial variation in chromosome number was obtained, and in the study on meiotic abnormalities, occurrence of structural aberrations was inferred.

S. officinarum × *S. robustum*

In a study of a *S. officinarum* (2n = 80) × *S. robustum* (2n = 80) hybrid, somaclones did not show much variation in morphology and numerical chromosomal variation. All the somaclones studied had 2n = 80 chromosomes. The F_1 hybrid by itself was cytologically very stable in root-tip mitosis and in meiosis. The lack of aneuploid chromosome variation in the somaclones can be attributed to the relative cytological stability of the donor parent (Jalaja and Sreenivasan, 1990b).

Conclusion

Somaclonal variation in sugarcane has a genetic basis, which in most cases can be cytologically proven. Recent molecular studies (Lu *et al.*, 1994) have indicated that most of the genetic variability seen in commercial cane varieties is due to the *S. spontaneum* chromosome complement maintained in the hybrid genome. The pre-existing chromosomal mosaicism and chromosomal aberrations, both structural and numerical, occurring during the tissue culture cycle, are the major reasons for the observed genetic variability in somaclonal populations of sugarcane. The ability of *Saccharum* species and species hybrids to tolerate both extensive chromosomal losses and gains, and the ability of deviant cells to differentiate into plants, makes the methodology useful in sugarcane improvement. With the standardization of techniques to cytologically detect chromosomal repatterning through *in situ* hybridization techniques (D'Hont *et al.*, 1995) it will be possible

to indicate precisely the extent of such genome repatterning. Future sugarcane varieties should have an in built mechanism to meet the challenges of water stress, salinity, alkalinity, diseases, and pests, and should be able to grow on marginal and sub-marginal lands. In this context sugarcane breeders will continue to transfer these desirable traits from wild species *S. spontaneum* and *Erianthus* through conventional breeding programmes. If judiciously used and thoughtfully integrated, somaclonal variation will certainly help the breeder to overcome some of the major problems in achieving desired goals through classical plant-breeding techniques.

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22. Field Performance of Selected Sugarcane (*Saccharum* spp. Hybrids) Mutants

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Introduction

Sugarcane belongs to the *Saccharum* genus, tribe Andropogoneae, family Gramineae. The *Saccharum* genus has five species: four wild species and the cultivated *Saccharum officinarum* L, 2n = 80. The wild species are:

1. *Saccharum barberi* Jerw. 2n = 40, 82, 92, 107, 116 and 124.
2. *Saccharum spontaneum* L. 2n = 48, 56, 64, 76, 80, 96, 112 and 128.
3. *Saccharum sinense* Roxb and Jeswiet. 2n = 106–120.
4. *Saccharum robustum* Brand and Jerw. (2n = 60, 70, 80, 82, 84, 90, 100, 118, 140, 144 and 148), is the ancestral species of *Saccharum officinarum* L. Both species, together with *S. spontaneum*, have given rise by crossing to the commercial varieties.

The basic number of chromosomes of the genus is 2n = 8–10, and the species previously described have been formed by ploidy.

The high ploidy of the species implicated in the genetic improvement, the instability of the chromosomes caused by aneuploidy (Price, 1962) and the chromosomal mosaics in this crop make basic genetic studies and improvement with classical methods very difficult. The efficiency of many crossing programmes is low, and 9–13 years of selection are necessary to manipulate large segregant populations.

Conventional Mutation Methods in Sugarcane

Sugarcane is one of the vegetatively propagated species which has been commonly used for conventional mutation research work. Studies were first initiated in Hawaii in 1929, and also in Taiwan in 1933. Mutation induction programmes have also been reported in Barbados, Fiji, India, Cuba and Jamaica. Even though physical methods were used as a general rule, chemical mutagens have also been employed. According to Micke *et al.* (1990) sugarcane is the species, within its group, with the largest number of varieties developed by induced mutations.

At the Institute of Plant Biotechnology (IBP), the use of somaclonal variation and *in-vitro* mutation induction, along with conventional mutation, has been an ongoing programme. The procedure entails using buds from 6-month-old plants, first immersed in an aerated NaN_3 solution (0.01%, pH 3.0). A population of 2000

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plants was obtained via this protocol and grown under natural rust (*Puccinia melanocephala* Sydow) infection conditions.

No resistant plants were selected in the plant cane or in the first ratoon. In the second ratoon a resistant stalk was selected and clonally propagated. The selected clone was highly resistant to rust and its agronomic characteristics were very similar to the original parental B 4362. Field trials were later done by comparing three rust-resistant somaclones versus a clone obtained via induced mutation in stalk buds all from variety B 4362. One of the somaclones was obtained by chemical treatment (NaN_3) of calli and two came from gamma-irradiated (Co 60) calli. The somaclones showed agronomic disadvantages and this limited their cultivation in commercial production. The major disadvantages were yield reduction, increased flowering and smut (*Ustilago scitaminea* Syd) susceptibility. The clone obtained by induced mutation in buds was selected as a commercial variety because of its outstanding agronomic traits.

Two thousand buds were treated with NaN_3 and in the second ratoon a resistant stalk was selected from among 50 000 stems (eight or nine stems per plant). This indicated that this clone developed as a result of breakdown of chimaerism.

The method employed required a high population; however, it was effective due to: (a) the fact that sugarcane has a high and fast germinating capacity; (b) rust in natural conditions has high infection efficiency and this makes possible to accomplish an efficient selection in 2-month-old plants. These results demonstrate that, for specific conditions, conventional mutation induction is effective.

In-vitro Culture as a Model for Mutation Induction: Chimaeras

The emergence of biotechnological techniques has enabled us to handle *in-vitro* organs, tissues and cells. This has opened up tremendous possibilities for their applications in massive plant propagation and genetic improvement.

Biotechnology did not escape to 'fashion' phenomenon, creating large perspectives in genetic improvement by somaclonal variation, indicated for the first time by Larkin and Scowcroft (1981). So far the expected results have yet to be achieved. This fact is reflected in the development of very few varieties, in spite of the large number of research projects. The advantage attributed to somaclonal variability are: the possibility of recovering the existing mutants in somatic tissues, and the novel mutants which cannot be produced either spontaneously or artificially (Evans and Bravo, 1986).

In practice, somaclonal variation has the following limitations:

1. Most of the mutants in sugarcane are aneuploids and/or polyploids (Heinz and Mee, 1969). Gene or point mutation frequency is low, of those that are more important, since in practical improvement only specific characters are required to change.
2. Novel mutants constitute a real fact, but their efficiency has not been demonstrated for most economically important characters.

3. For *in-vitro* culture other causes of variability arise together with mutations, which in regenerated sugarcane plants show high frequency than real mutations (Pérez Ponce, 1987). Epigenetic effects and juvenile stage can provoke these changes, and there were many projects in which somaclonal variability has been over-estimated.
4. In tissue culture, plant regeneration from cells or a group of cells, will affect the chimaerical constitution of the obtained plants. However, it is important to indicate that, in spite of the plant origin from a single cell, regeneration takes place *in vitro*. Hence other factors that affect variability are bound to have an effect, so the problem of chimaeras will always be present.

In-vitro *Culture as a Model for Mutation Induction*

As previously indicated, the use of somaclonal variation for genetic improvement has limitations. Now we will look at the possibilities of *in-vitro* culture application as a model for induced mutation studies.

The great theoretical and practical advantages of *in-vitro* cultures are based on the principle of totipotency; cells can be considered as individuals, and this has the following advantages:

1. Mutagen treatments can be applied at the Petri dish level, increasing their efficiency and the treated population.
2. *In-vitro* selection has a great advantage at the cellular level.
3. With the exception of conventional mutation induction in pollen or ovaries, *in-vitro* culture offers the possibility of reducing chimaera frequency when plants are regenerated from a single or few cell aggregates.

The ideal approach would be the use of *in-vitro* mutation induction followed by *in-vitro* selection at the cellular level, by using cell suspensions and regenerating plants to eliminate or minimize somaclonal variation, epigenetic changes and chimaeras to a large extent

To approach the proposed model in sugarcane, we accomplished the following:

1. Employment of culture media with minimal hormonal (auxins) and high concentrations of coconut milk (18%) for callus induction. With this culture medium, chlorophyl and morphological changes *in vitro* have been reduced to more than half, compared with the use of culture medium proposed by Heinz and Mee (1969).
2. It is demonstrated that *in-vitro* age influences somaclonal variation and epigenetic changes in regenerated plants. For this reason, physical mutation treatments are given to 2–3-month-old callus, depending on the regeneration capability of the cultivars.
3. Radiation doses not exceeding 5–20 Gy are applied; this is below those reported by most authors. This is to reduce the frequency of chromosomal mutations and to increase the frequency of mutations involving single or few genes (Perez Ponce, 1987).

Chimaeras

Chimaeras cause many obstacles in the selection process, and they have been the subject of investigations. Table 1 indicates the chimaera frequency depending on the regeneration pathway. Results clearly show that it is possible to reduce chimaeras in tissue culture. This is a simple method in which an epidermis coloration marker is employed

Seven-month-old calli of variety My 5450 were treated with 30 Gy (Co 60) during growth and regeneration stages. Regeneration initiated when purple and green points appeared on the callus surface 5 or 7 days after placing the callus on the regeneration medium. Morphological characters in regenerated plants were evaluated according to van Dillewijn (1952). The results of this experiment showed the problems of selection in *in-vitro*-regenerated populations: the high variability observed in vitro in plants, gradually disappear as clonal multiplication cycles progress. For chimaeras, the phenomenon is maintained with the same trend (Table 2).

Mutation induction treatments increased variability mainly when they were accomplished at the regeneration stage. The disadvantage of mutagenic treatments

Table 1. Chimaera frequency related to multiplication systems (Gerth, 1991)

Multiplication system	No. of evaluated plants	Variegated	Non-variegated
Original clone (C 8751)	370	200	170
Bud multiplication	5650	4831	846
Callus culture	3478	0	3478
Rhizome	68	64	4
Apex culture	136	45	91
Meristem culture	132	11	121
Cell suspension	22	0	22

Table 2. Chimaera frequency depending on the stage (callus multiplication or plant regeneration) where irradiation (Co 60) is applied

		Plant cane		First clonal multiplication	
Dose (Gy)	No. of regenerated plants	Percentage total changes	Percentage of chimaeras	Percentage total changes	Percentage of chimaeras
0	153	8.50[c]	2.62[c]	1.31[c]	1.31[c]
Callus growth, 30	162	29.61[b]	9.38[b]	11.05[b]	5.55[b]
Regeneration, 30	173	60.10[a]	33.53[a]	21.40[a]	10.41[a]

[a, b, c]Different letters differ statistically for the Duncan test.

at the regeneration stage is the increase in chimaera frequency. This phenomenon was proportionally similar to the chimaera frequency when calli were irradiated at the growth phase.

Managing *In-vitro*-regenerated Populations

Estimation of In-vitro *Variability*

For practical improvement, variability estimation is necessary to determine the mutagenic treatments to be applied. Chlorophyll deficiencies have been used as variability markers in conventional mutation induction. To estimate the frequency of chlorophyll and morphological changes *in vitro*, the classification of Gustaffson (1940) and Kalan and Orav (1974) were used. Somaclonal variability was first studied and differences among cultivars were found. Cultivar CP-5243 showed the highest values (20.15–41.50%), followed by My 5450 with 15.94–21.33% and the variety B 4362 with least changes, 3.52–12.20%.

Upon evaluating the effect of mutagenic treatments in three mentioned varieties (applied dosage 10–50 Gy (Co 60)), a definite trend between the frequency of changes *in vitro* and the variability in field conditions was not found. In this project more than 15 000 plants *in vitro* and 5000 plants in the field were evaluated. These results showed that the chlorophyll changes are unreliable in determining genetic variability, because of the different factors that affect the plant regeneration process.

Many factors, such as genotype, culture media, callus age and mutagenic doses must be analysed. For example in the previous work, radiation doses below 10 Gy (Co 60) did not increase the changes *in-vitro* with respect to somaclonal variation; however, this dose has been effective for genetic improvement.

To estimate somaclonal variation and the rate of induced mutation frequency, the morphological classification according to van Dillewijn (1952) was employed (Table 3). These results showed that mutations did not cause high variability, but epigenetic and juvenile effects. The over-estimation of the variability observed on *in-vitro*-cultured plants has led to wrong thinking about the possibilities on the use of somaclonal variation for crop improvement. Despite the number of research papers dealing with the use of somaclonal variation for genetic improvement and the high variability reported in most of them, there are only a few practical results.

It is important to know the vegetative multiplication cycle for a reliable estimation of the qualitative characters. There is a considerable decrease in variability in the first ratoon (Table 3) with a significant difference between somaclonal variation and induced mutations. Then, there is a certain degree of reliability in the multiplication cycle.

When these changes were evaluated till the third clonal multiplication, there was a decrease in total variability; however, there were differences between somaclonal variation and mutagenic treatments. This behaviour has been observed

Table 3. Influence of somaclonal variation and mutation induction in qualitative changes

Dose (Gy)	Cycle	No. of plants	Qualitative changes (%)											Test of Significance	
			Dewlap	Auricle	Ligule	Aerial shoots	Aerial roots	Tillering form	Leaves	Nuts	Root rings	Others	Total	Plant cane	Ratoon
0	Plant cane	224	4.02	8.03	3.58	6.69	3.57	2.24	4.46	3.12	4.46	0	40.17	b	
	Ratoon	224	0.92	1.40	0.92	0	0	0.92	0.92	0.47	0.47	0	6.02		c
10	Plant cane	214	4.67	2.80	2.33	11.21	9.34	6.54	4.67	4.67	3.27	1.40	50.93	a	
	Ratoon	206	1.94	0.97	1.46	0.97	0.49	2.91	0.97	0.97	0.97	0.49	12.14		b
50	Plant cane	188	5.32	4.25	3.19	7.45	9.57	5.32	2.66	2.66	3.19	1.59	45.21	a	
	Ratoon	152	3.95	1.97	5.26	1.31	1.97	5.26	1.97	1.31	1.97	1.31	26.32		a

(variety CP 5243, 4-month-old calli, Heinz and Mee (1969) culture media)
[a, b, c]Different letters differ statistically for the Duncan test 5%.

in all the experiments conducted, which demonstrates that most of the variation in the regenerated plants is not of mutational origin.

With respect to variability estimation in qualitative characters, our results indicated that it is possible to estimate with a certain reliability in the first ratoon, and from the third clonal multiplication the remaining changes have a high probability of being real mutations. This means that direct evaluation of vitro plants is a big mistake.

Selection of Quantitative Characters

For practical improvement, quantitative traits are of greater economic importance. In addition to the already explained factors that influence variability, these characters are also influenced by the environment as another cause of variation. The question further arises: which clonal generation is ideal for effective selection for the main yield components? To answer this question we analysed the behaviour of micropropagated plants.

Field studies with plants regenerated via axillary bud proliferation helped us to determine the changes or temporary modifications induced by *in-vitro* culture on the principal yield components in the vitro plants and their subsequent clonal multiplications.

The main characteristic of *in-vitro*-propagated plants was the increase in the number of stalks, between 15% and 50% depending on the genotype as compared with plants propagated from conventional seedstalks. Flynn and Anderlini (1990) and Feldmann *et al.* (1994) further indicated that this effect could be maintained after two vegetative multiplications in the field where a 12% increase in stalk density per area was still observed. Therefore, the number-of-stalks trait should not be selected before three vegetative propagations of the plants regenerated *in-vitro*.

As a result of an increase in the number of stalks, a decrease in the diameter and weight of stems was observed. These two variables, by their correlation with population density, should not be selected until the third clonal multiplication. However, the genotypic influence is notable in the relationship between these characters.

Since there is a decrease in the weight of the stems in the micropropagated plants as compared to seedstalks, all genotypes do not show similar behaviour. There exists a significant interaction between the varieties and the propagation method (Table 4). Genotypes CP 5243, UCLV 84–159 and UCLV 83–86 showed a significant decrease in the weight of stems, while genotype UCLV 84–103 indicated no statistical differences between micropropagated plants and traditional seed.

This interaction is better expressed upon analysing the relationship between the increase in the number of stems and the decrease in weight (Table 5). The genotype UCLV 84–103 is capable of assimilating a greater increase in stalk density without a meaningful decrease in weight, while UCLV 84–159, with a small increase in the number of stems, presented a greater reduction in weight. This behaviour is determined by the growth habits of the varieties.

Table 4. Interaction of genotype × multiplication method (conventional seedstalks/micropropagation) for stalks weight (Kg)

CP-5243		UCLV 84–103		UCLV 84–159		UCLV 83–86		
Micropro-pagation	Stalks	Micropro-pagation	Stalks	Micropro-pagation	Stalks	Micropro-pagation	Stalks	
1.19^{b}	1.62^{a}	1.22^{b}	1.29^{ab}	0.94^{c}	1.34^{ab}	0.85^{c}	1.13^{b}	$x = 1.20$; E.S. = 0.06*

Significance 5% Duncan test.

[a, b, c]Different letters differ statistically for the Duncan test.

Table 5. Effect of genotype and relationship between number of stalks and stalk weight in micropropagated plants and cane stalks

Variety	Propagation method	No. of stalks/m	Percentage stalk increase	Stalk weight (Kg)	Percentage weight decrease
UCLV 84–103	Micropropagation	19.4	35.7	1.22	5.4
	Seedstalks	14.3		1.29	
UCLV 84–86	Micropropagation	25.2	50.0	0.85	24.8
	Seedstalks	16.8		1.13	
UCLV 84–159	Micropropagation	17.6	13.5	0.94	29.8
	Seedstalks	15.5		1.34	
CP 5243	Micropropagation	21.1	43.5	1.19	26.5
	Seedstalks	14.7		1.62	

In-vitro-regenerated plants showed reduced flowering. The high-flowering variety CP 5243 did not show inflorescences during the growing cycle; low-flowering frequency was observed in the first clonal multiplication (9–11%), and in the second one the normal flowering capacity (80–100%) was re-established.

Sugar content and stalks height did not differ in plants propagated by conventional seed. The sugar content is a very stable character, and is possible to select in seedling stage, directly in vitroplants. *In-vitro*-regenerated plants are highly susceptible to rust (*Puccinia melanocephala* Sydow) (Peros and Bonnel, 1990). This susceptibility can be associated with temporary changes in the anatomical structure of the leaf that favour the pathogen infection process. However, after re-establishment of the normal structure of the leaf during field growth, plants recover their normal resistance level. In our experience, micropropagated plants, of most of the genotypes present two degrees more susceptibility than the seed-

stalks, on a scale 0–5 proposed by Alfonso and González (1979). Nevertheless, it is possible to screen for rust resistance among *in-vitro*-regenerated plants, since in spite of showing a greater susceptibility to the pathogen, genotypic differences are clearly expressed. To overcome this drawback, micropropagated plants from resistant and susceptible genotypes as well as the original variety are included as control in plots or selection growth beds. Thus, it is possible to select mutants showing a similar response to micropropagated resistant genotypes. Using this methodology, three rust-resistant somaclones from the susceptible variety B 4362 have been obtained.

The age of *in-vitro* cultures is another factor that influences the magnitude of the changes or temporary alterations that are expressed in the regenerated plants. As *in-vitro* cultures become older, variation in plant populations tends to increase, not only in real mutants due to aneuploids or polyploids, but also in epigenetic or physiological modifications.

The effect of the age of *in-vitro* cultures was studied in micropropagated plants from 4 to 20 subcultures on the proliferation medium (0.3 mg/l BA). Plants with longer time culture *in vitro* showed a greater stimulation in the number of stalks per area and a reduction in stalk diameter, in comparison with plants from only four subcultures and conventional seed (Table 6). In addition, a reduction in stalk height and a decrease in sugar content was observed. The differences between *in-vitro* treatments disappeared after vegetative multiplication of the vitroplants.

This effect could be related with the behaviour observed *in-vitro*, characterized by an increase in the number of shoots per explant with the age of the cultures (Jiménez, 1995). This is a result of the habituation effect of tissues grown for long periods in cytokinin-containing media or high juvenile stage induced by prolonged *in-vitro* culture conditions. The changes or modifications previously described affect all plant populations, and result from physiological disorders induced by duration of *in-vitro* cultures and/or the extended exposure to cytokinin-containing media. However, there are qualitative changes that appear with a given frequency with *in-vitro* age; e.g., grassy plants with a large number of stems with reduced height and diameter and poor root development.

Table 6. Influence of number of subcultures on plant cane

	Stalk height *(cm)*	Stalk *diameter (mm)*	No. tillers/m	Brix
Control	264.2[a]	27.0[a]	11.6[c]	22.9[a]
4 Subcultures	262.4[a]	25.6[b]	14.7[b]	22.0[a]
20 Subcultures	238.0[b]	23.9[c]	16.0[a]	20.9[b]
	x = 254.8; E.S. = 5.53***	x = 25.5; E.S. = 0.50**	x = 14.1; E.S. = 0.25***	x = 21.9; E.S. = 0.32*

*5% significance; **1% significance; ***0.1% significance

[a, b, c]Different letters differ statistically for the Duncan test.

The frequency of this phenomenon is also influenced by the genotype. With 16 subcultures, in the variety CP 5243, 50% grassy plants were observed, while in the variety C 8751, the frequency was only 1%. Vegetatively propagated plants recover their normal development, which is evidence for the epigenetic cause of these changes. Therefore, for *in-vitro* improvement, the number of subcultures must be minimized. This is done to maintain the regeneration capacity when callus or cell suspensions are employed, and to minimize the temporary modifications, physiological disorders or epigenetic changes associated with age *in-vitro*, which are undesirable in the selection process.

In-vitro Selection

More than 20 years ago Carlson (1973) demonstrated for the first time that cells and plant protoplasts could be selected against pathogen toxins, and regenerated plants obtained with changes to the infection response by the pathogen. Since then a large number of assorted trials have been accomplished concerning the vast potential of selection, using cell culture for the development of new disease-resistant genotypes.

It is essential that *in-vitro*-selected traits are expressed at the cellular level, e.g. distinction between resistant and susceptible cultivars. When this requirement is fulfilled, the effect of the pathogen or other adverse condition could become the target object of *in-vitro* selection. On the other hand, there must be a direct correlation between the results of *in-vitro* selection and plants in the field.

Practical examples on the use of *in-vitro* selection to obtain disease-resistant somaclones of sugarcane have been reported by Heinz *et al.* (1977); to *Helmisthosporium sacchari* (Maribona *et al.* (1996) and *Ustilago scitaminea* Syd. (Gómez (1996) For the development of this work, the first step was to standardize the culture medium for fungal growth. The culture medium contained the inorganic salts of Murashige and Skoog (1962) modified with 2% sucrose in liquid state.

The next step was to determine the concentration of the selective agent employed for selection in variety Ja 60–5. Calli of this cultivar were used because it is a recalcitrant genotype. There was an increase in callus mortality (degrees 1 and 2 of the scale) at the highest dose of the crude extract (Table 7). Ninety-three per cent mortality was reached with 87.5 ml crude extract per litre in the culture medium, which is an acceptable value for selection process taking into account, that for a better and reliable selection, a high percentage of callus exposed to selective agent should die. This would indicate the possibility for cells or groups of cells (mutants) to express resistance.

After the evaluation of the plant canes from first and second ratoon in the *in-vitro* selected population, a correlation between cell level expression *in-vitro* and the behaviour of plants in the field was confirmed. This is a primary concern to use *in-vitro* selection method in the genetic improvement programme.

A large percentage of *in-vitro*-selected plants (52 and 46% in plant cane and ratoons) demonstrated resistance in the field when compared with the use of so-

Table 7. Effect of *Ustilago scitaminea* Syd crude extract on callus growth (variety Ja 60-5)

Concentration (ml/l)	Degree of callus growth (%)*					Mean of calli growth	Significance (1%)
	1	2	3	4	5		
0	0	2.20	6.70	13.30	77.80	4.70	a
20.92	22.0	26.0	28.0	14.0	10.0	2.64	b
50.83	48.0	14.0	12.0	16.0	10.0	2.60	b
87.5	85.0	8.0	6.0	4.0	2.0	1.50	c

*Callus growth was evaluated according to the methodology proposed by Santana (1982); Heinz and Mee (1969) culture media.

Degree 1 = dead callus; degree 5 = 100% callus growing area.

[a, b, c]Different letters differ statistically for the Duncan test.

maclonal variation and induced mutations without using a selective agent (5.10% and 4.08% in ratoons 1 and 2). Thus, the number of *in-vitro*-selected plants to handle in the field is smaller, with a greater percentage of success.

Out of 113 *in-vitro*-selected plants, two resistant somaclones were obtained (UCLV 89–163 and UCLV 89–169). These results demonstrated the possibility of obtaining resistant genotypes by exploiting somaclonal variation when an efficient selection system at the cellular level was established.

Using *in-vitro* mutagenesis and early stage selection with fungus inoculum on roots (Orellana and Pérez Ponce, 1987), 15 000 plants were evaluated, and so far no resistant somaclones have been selected. This demonstrates that mutation frequency for gene(s) involved in smut resistance is low, and also becomes more complicated by the high level of ploidy in the species. However, it is possible to obtain smut-resistant somaclones (Chen *et al.*, 1978; Liu, 1981; Ferreira, 1986; Sreenivasan *et al.*, 1987; Pérez Ponce *et al.*, 1993), but, there were failures as well. Daub (1986) reported more than 30 000 somaclones from six susceptible cultivars without having selected resistant plants. To begin with, cultivars with some level of resistance have the possibilities of greater success. However, not all the selected somaclones were useful from an agronomic point of view (Ferreira, 1986; Daub, 1986). Another problem in the use of somaclonal variation is the high frequency of aneuploids and polyploids. Thus, linked with the resistance trait, some agricultural disadvantages arise. As an example, somaclone UCLV 89–169 with smut resistance obtained by *in-vitro* selection, was eliminated because of its fine stalks.

When Larkin and Scowcroft in 1981 outlined the somaclonal variation concept, great expectations were raised in utilizing the system for crop improvement. However, there are few successful results (Van den Bulk, 1991). This is due to the complexity of selection in *in-vitro*-regenerated populations. This was amply demonstrated by the fact that, until five clonal multiplications of the plants had been made, effective selection for smut resistance could not be accomplished.

Originally all the somaclone population showed smut resistance during the first three multiplication cycles, and on fourth multiplication, four of 20 somaclones were susceptible; on fifth multiplication, only two somaclones maintained resistance against the pathogen. This demonstrates the complexity of the selection process, and the contradictions and false expectations while using this technique.

However, the direct *in-vitro* selection method for smut resistance (Table 8) reduced the plant population by eliminating most of the susceptible plants during the first stage of selection. This is of tremendous importance in handling a large number of plants. In addition, there is increased efficiency, since the selection is accomplished directly or indirectly at the cellular level either in callus or cell suspension.

Use of Somaclonal Variation and Induced Mutations for Genetic Improvement

As mentioned earlier, even when *in-vitro* selection could not be achieved, *in-vitro* mutagenesis has other advantages. When selection is accomplished *ex vitro*, it is necessary to work with large mutant populations, and for specific characters such as disease resistance, screenings can be done in greenhouses or growth beds (early stages) where it is possible to handle large populations with optimum pathogen inoculation conditions.

In sugarcane, the principal phytosanitary problems are rust (*Puccinia melanocephala* Sydow), smut (*Ustilago scitaminea* Syd.), and sugarcane stalk borer (*Diatraea sacharalis* Fabricius).

The strategy for selection against rust resistance is to apply mutagenic treatments in stalk buds or callus and select at the early phase (growth beds). For smut

Table 8. Protocol for *in-vitro* selection for smut (*Ustilago scitaminea* Syd) resistance in sugarcane

	Stage	Months
I	Callus growth and *in-vitro* culture of the pathogen	1
II	Callus multiplication and production of crude extract	1
III	*In-vitro* selection (two selective subcultures)	2
IV	Plant regeneration	1
V	Rooting	1
VI	Root inoculation and early stage selection in greenhouse	3
VII	Transplanting to field conditions and four clonal multiplications every 6 months to stabilize the changes produced by tissue culture in plants	24
VIII	Field trials for agronomic characters and disease resistance; seedstalk inoculation with fungus teliospores	12
IX	Field trials for agronomic characters and disease resistance; ratoon	12
	Total	57

resistance, mutation, in callus and cell suspensions is combined with *in-vitro* selection. Sugarcane stalk borer resistance can be developed by genetic transformation with *Bacillus thuringensis* genes (Arencibia *et al.*, 1995).

Flowering capacity and growth habits are two other aspects that limit the use of some good cultivars in commercial production. Flowering is important because of the narrow margin for harvest and the sugar losses it causes. Growth habit includes several aspects that are not always evaluated correctly, resulting in problems for mechanical harvesting and lowering the photosynthetic efficiency when the stalks are logged. This is not always taken into account.

With respect to yield components, because of their genetic complexity, interesting results should not be expected. Nevertheless, it has been possible to obtain somaclones of interest for disease resistance, high yield, sugar content or flowering reduction (Muller, 1985; Liu and Hsieh, 1986; Pérez Ponce *et al.*, 1993). Table 9 summarizes the results from the sugarcane improvement programme carried out at the Institute of Plant Biotechnology (IBP). The following conclusion can be drawn from Table 9: genetic improvement by mutation induction in callus is more efficient than hybridization, which requires a large plant population and selection cycles last for nearly 13 years. Because only few changes are introduced, it is possible to take advantage of the well-known desirable traits of the variety to be improved.

Rust resistance in B 4362 cultivar has been one of the most-studied characters. Fifty thousand plants were evaluated using somaclonal variation as the only source of variation, but without success (Maribona, personal communication). Similar results were reported by others. As shown in Table 9, three rust-resistance somaclones were obtained. However, they were all agronomically inferior when compared with the clone obtained by induced mutations of buds.

Table 9. Results of mutation induction programme at the Institute of Plant Biotechnology

Character to be improved (genotype)	Mutation induction treatment (explant)	No. of regenerated plants	Selection		Released varieties
			Percentage of selected plants	No. of selected somaclones	
Flowering reduction (CP 52-43)	10–30 Gy, callus	5000	0.16	8	1
Sugar content (POJ-2878)	5–20 Gy, callus	5000	0.08	4	2
Rust resistance (B-4362)	10–40 Gy and NaN_3 0.01%, callus	22 928	0.02	3	0
Growth habit (Ja-60-5)	10–20 Gy, callus	6000	0.016	1	1
Smut resistance (My5450, Ja-60-5)	10–30 Gy, callus	15 500	0	–	0
Total		53 928	–	16	4

Rust is an obligate parasite; therefore, *in-vitro* selection is not feasible due to the inability of *in-vitro* culture of the pathogen. Nevertheless, it is possible to use induced mutations for rust resistance. However, to select in vitroplants at the early stages, the juvenile effect of *in-vitro* culture, which increases rust susceptibility, must be taken into account.

CP-5243 mutants with flowering elimination or reduction (less than 15%), showed an increased yield due to a rise in stalk height (an important component of yield). The yield increase was caused by an increase in the height of the stalks by about 20%. This yield improvement can be considered a result of pleiotropic effect.

The cultivar POJ-2878 saved the sugar industries of the world due to its virus resistance. This is the only variety that was eliminated from commercial production without being severely affected by diseases. To rescue this variety, a clonal selection was done, and the best seven clones were employed for mutation induction. Two selected somaclones developed into commercial cultivars, and have a increase in sugar content.

These results corroborate the possibilities of *in-vitro* mutagenesis for the genetic improvement of sugarcane. When *in-vitro* selection can be accomplished, with induced mutations, the effectiveness of the technology is increased. By combining biotechnology and mutation induction, we enter a new era of mutation genetics, which has greater potential in sugarcane improvement than in any other crop because of its polyploid and heterozygous constitution.

Conclusions and Future Prospects

In spite of the high polyploid level of sugarcane, conventional mutation induction has been effective to obtain improved clones. Even though, there are no comparative experimental studies, somaclonal variation has been less effective in sugarcane than *in-vitro* mutagenesis. By *in-vitro* mutagenesis 53 928 plants were evaluated *ex vitro*, and 16 were selected with improved characters such as rust resistance, smut resistance, sugar content and flowering reduction. This gave a frequency of one useful mutant every 3370 individuals. From these 16 variants, four are commercial cultivars, with a frequency of one cultivar for each 13 482 plants. This value is below the number of individuals studied in most hybridization programmes.

Resistance to diseases such as smut (*Ustilago scitaminea* Sydow) and eye spot (*Helminthosporium sacchari*) can be selected *in vitro*. One hundred and thirteen plants were obtained by *in-vitro* selection against smut resistance, and two improved somaclones were selected. One of them has undergone field trials and will be released as a commercial variety very soon.

In sugarcane, the lack of knowledge and the empirical nature of most hybridization programmes means that the high-yielding genotypes occur with a low frequency. There are few cultivars that, in addition to yield, comply with other re-

quirements, such as resistance to biotic and abiotic stress, growth habit, flowering and growth cycle.

Induced mutations have an added advantage in combination with plant tissue culture and can become a useful tool to correct those defects which limit the commercial use of new varieties or clones generated by hybridization. Hybridization is essential for genetic improvement, since gene combination and recombination by crossing is, and will be, the fundamental source of variability for selection. Hybridization programmes will have to be run alongside those that employ biotechniques such as *in-vitro* mutation, molecular biology and genetic engineering.

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SECTION 3

23. Molecular Basis of Heritable Tissue Culture-induced Variation in Plants

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Introduction

Plant tissue culture techniques are used extensively in the propagation of ornamental and horticultural plants, in biochemical and molecular studies of plant cellular processes, and in plant transformation protocols. Genetic identity among cells or plants is usually desired or assumed in these experiments. However, the tissue culture process does not always produce clonal cells or plants, and often results in numerous morphological changes (reviewed in Sunderland, 1973; D'Amato, 1977, 1985; Bayliss, 1980; Larkin and Scowcroft, 1981, 1983; Orton, 1984; Ahloowalia, 1986; Larkin, 1987; Lee and Phillips, 1988; Sun and Zheng, 1990; Peschke and Phillips, 1992; Kaeppler and Phillips, 1993a).

Tissue culture-induced variation is manifested in three ways. The first type of variation is within the culture itself. Individual cells within the culture may vary in morphology and genetic make-up. This variation is of most relevance in transient expression where cultured cells are used to assess biochemical or physiological processes. Much of the variation present in cultures is probably not recovered as regenerated plants, since changes within cells could reasonably be expected to affect totipotency. Second, primary regenerants can show a non-heritable phenotypic effect of the culture process. This type of variation is not sexually transmissible and is of most relevance in culture systems where the asexually propagated plant is the final product of the culture effort. This variation may be propagated asexually through cuttings or grafting but may show somatic instability. The third type of culture-induced variation is sexually transmissible mutation or epi-mutation, which can be heritably transmitted from regenerated plants to their offspring. Heritable variation is of most importance when the end product is seed-derived plants. An example would be variation resulting from tissue culture/transformation efforts in agronomic crops such as maize, oat, and soybean.

Efforts to understand the molecular basis of tissue culture-induced variation have led to an extensive documentation of types of variability induced by tissue culture, but have failed to produce satisfactory answers describing why tissue culture is mutagenic. It is reasonable to suggest that one or two cellular processes or mechanisms may be responsible for the extensive array of variation found in tissue cultures, and in regenerated plants and their progenies. These underlying

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processes or mechanisms hold the key to elucidating tissue culture-induced change and the reason for its occurrence.

This chapter will focus specifically on heritable variation among regenerant-derived progenies. Types of mutagenesis and their potential importance in tissue culture-induced variation will be described. Specific emphasis will be placed on possible underlying mechanisms resulting in mutagenesis, and the potential to use these hypotheses to better understand why tissue culture is mutagenic.

Documented Types of Phenotypic Variation

Qualitative Trait Variation

Qualitative trait variation is among the most frequently observed types of mutation found among progenies of regenerated plants and has been observed in several species (reviewed in Larkin and Scowcroft, 1981, 1983; Orton, 1984; Ahloowalia, 1986; Larkin, 1987; Sun and Zheng, 1990; Peschke and Phillips, 1992; Kaeppler and Phillips, 1993a). Qualitative mutations are especially frequent and striking in species such as maize, which is diploid and exhibits extreme morphological phenotypes when certain genes are inactivated. Several trends are evident from analysis of qualitative mutation frequency. Data from maize will be used to illustrate these trends.

Mutation Frequency and Effect of Culture Age

Mutations in tissue culture occur frequently based on the number of qualitative mutants observed in maize (summarized in Kaeppler and Phillips, 1993a). After only 4 months in culture, regenerated plants contained on average 0.52 heterozygous qualitative mutants per plant (Lee and Phillips, 1987b). This frequency would be many orders of magnitude less in seed-derived plants. In the same study, cultures maintained for 8 months produced regenerated plants containing on average 1.32 heterozygous mutations per plant. The increased frequency of mutations over time demonstrates the culture age effect. Studies in rice (Fukui, 1983) and maize (Zehr *et al.*, 1987) have shown that this age effect is primarily due to a sequential accumulation of mutations over time, rather than an increased mutation rate in older cultures.

Effect of Genotype

Variability for tissue culture-induced mutation frequency among species or among genotypes within a species has been shown in several studies (Liu and Chen, 1976; Edallo *et al.*, 1981). Zehr *et al.* (1987) showed variation among seven maize genotypes for the frequency of segregating qualitative mutations. McCoy *et al.* (1982) reported differences in the frequency of chromosomal abnormalities

among regenerants from the oat cultivars Tippecanoe and Lodi. After 4 months in culture, 49% of Lodi regenerants showed chromosomal abnormalities compared to 12% of Tippecanoe regenerants.

Plant Sectoring

Regeneration of plants in most species is often from cell aggregates, rather than single cultured cells. It appears that plants regenerated from embryogenic cultures are derived from a smaller number of cells than plants regenerated from organogenic cultures. The multicellular origin of regenerated plants has been shown by sectoring for qualitative mutants (Armstrong and Phillips, 1988). This is especially evident in monoecious species such as maize where the separation of the floral structures can result in the tassel being genetically different from the ear. This chimaerism leads to segregation of mutants in the second selfed (sexual) generation after plant regeneration. This observation not only illustrates the multicellular origin of regenerated plants, but also reflects the genetic variability among cell clusters within a culture.

Stability of Mutations

Qualitative trait mutations induced by the tissue culture process are most often recessive and generally appear to be stably inherited. In most cases the variants segregate as single gene mutations and appear reliably in the sexual generations studied. Some exceptions do exist, but can be considered infrequent. These exceptions include somatic instability (Groose and Bingham, 1986), and loss of the mutated phenotype upon outcrossing (Oono, 1985). These exceptions are discussed in more detail in the ensuing section on mechanisms of mutation.

Quantitative Trait Variation

Quantitative trait variation is frequently observed among tissue culture-derived plants. Quantitative trait variation, by definition, is more difficult to observe visually than qualitative trait variation and has, therefore, been less frequently reported. However, variation is frequently found in instances where quantitative traits have been measured (Earle and Gracen, 1985; Zehr *et al.*, 1987; Lee *et al.*, 1988; Carver and Johnson, 1989; Dahleen *et al.*, 1991).

Table 1 provides data illustrating quantitative variation for various agronomic traits among regenerant-derived B73 maize lines. Consistent with other studies, the trend is towards poorer performance and smaller plant stature. The data shown include both regenerant-derived lines *per se* as well as crosses of the regenerant-derived lines to the non-cultured source inbreds. Performance of the hybrid plants is always better than the regenerant-derived lines *per se* and approaches the performance of the non-cultured source inbred. These observations indicate that tissue culture-induced quantitative mutations show additive to recessive gene

Table 1. Agronomic performance data for 26 regenerant-derived maize lines and the non-cultured control inbred (B73). Original R_0 plants were regenerated after 3 months in culture. The experiment was grown in three locations in 1990 and 1991. Two replications were grown at each location.

Class	*n*	Moisture (mg/g)	Yield (Mg/ha)	Plant height (cm)	Ear height (cm)
Control	3	322a*	2.73a	194a	96a
Regenerant-derived lines					
Selfed lines	26	288c	2.41c	187d	93c
Lines × control	26	292b	2.70a	192b	97a
Lines intercrossed	26	289c	2.57b	188c	94b

*Letters indicate significance at $\alpha = 0.05$.
mg/g = milligram of water per gram of weight of kernel; Mg/ha = Megagrams per hectare.

action, and can be interpreted at the individual locus level as a loss or reduction in gene function of the involved loci.

Documented Types of Molecular Variation

Molecular variation has been characterized at both the DNA and protein level using a number of different approaches. In many instances, the observed variation is a DNA or protein electrophoretic band of altered mobility or a chromosome of altered structure. As with phenotypic mutations, the exact base sequence alteration is often unknown. This section of the review provides examples of the types of molecular variation characterized and, while not exhaustive, summarizes the types of variation observed.

Variation at the Chromosome Level

Karyotype variation has been observed in cultures, regenerated plants, and progeny of regenerated plants in numerous species (Ahloowalia, 1975, 1983; Bayliss, 1980; Edallo *et al.*, 1981; McCoy and Phillips, 1982; Armstrong *et al.*, 1983; Karp and Maddock, 1984; Benzion *et al.*, 1986; Johnson *et al.*, 1987; Lee and Phillips, 1987a; Masound *et al.*, 1991). Types of altered karyotypes include chromosomal rearrangements as well as aneuploidy and changes in euploid levels.

Changes in chromosome structure observed in regenerated plants and their progenies include translocations, inversions, deletions, and duplications. These aberrations often result in pollen sterility of the primary regenerant. Pollen sterility has served as a useful tool in screening regenerated plants for putative chromosome rearrangements. Many chromosomal aberrations are not transmitted to the progeny because of this gametophytic screen and must be observed in the primary regenerants.

Ploidy level variation has been documented in several studies of cell cultures and has been observed in regenerated plants and their progenies. Severe aneuploidy affects the ability of cells to regenerate into plants. Should the plant regenerate, the aneuploid condition is often not transmitted, especially in diploid species. Polyploidy may also occur in cultures and it is possible that this condition would be found in regenerated plants and transmitted to their progeny. In general, changes in chromosome number are less frequently detected than other karyotypic aberrations.

An example of the relative frequency of various chromosome aberrations is summarized in Kaeppler and Phillips (1993a). Data from four separate studies in maize show that chromosome structural variation occurrs four times more frequently than chromosome number variation in primary regenerants. The trend in oat was similar with structural variants also four times more frequent than changes in chromosome number.

Chromosome breakage events in these studies were usually associated with centromeric or interstitial heterochromatic blocks (Lee and Phillips, 1987a; Johnson *et al.*, 1987). This observation led to the hypothesis that chromosome aberrations in culture are due to late replication of heterochromatin in culture resulting in chromosome breakage events (Lee and Phillips, 1987a). The hypothesis is based on precedent described by Rhoades and Dempsey (1972, 1973) explaining breakage events in 'high-loss' genotypes of maize.

Variation at the DNA Level

Variation at the DNA level has been most extensively characterized using restriction enzyme analysis. Studies using methylation-insensitive enzymes (specifically, insensitive to CG or CXG methylation) produced restriction length polymorphisms in a number of different studies (Roth *et al.*, 1989; Brown *et al.*, 1990, 1991; Müller *et al.*, 1990). In most cases, changes in the restriction pattern appeared to be due to altered fragment size, rather than additions or losses of restriction fragments. Investigation in soybean showed that all restriction fragment length alleles identified in regenerant-derived plants could be found in other soybean cultivars (Roth *et al.*, 1989).

Studies using restriction enzymes sensitive to 5-methylcytosine modification have found extensive and frequent change in restriction fragment length patterns (Brown *et al.*, 1990, 1991; Kaeppler and Phillips, 1993b). Recent results in maize (Olhoft, 1996) provide an example of the nature of methylation variants among regenerant-derived progeny. The study involved methylation analysis of 206 single copy sequences in 21 regenerant-derived families. Decreases in methylation occurred three times more frequently than increases in methylation. Methylation change was much more frequent than sequence change based on the comparison of *Hpall* vs. *MspI* restriction digest polymorphisms. These results exemplify trends that we have observed (see also Kaeppler and Phillips, 1993b) although some reports indicate equal frequencies of methylation and sequence variation (Brown *et al.*, 1991).

Variation at the Protein Level

Variation has also been characterized for several types of proteins with grain storage proteins and isozymes being most frequent. Characterized variation can be summarized into three categories: altered electrophoretic mobility, loss or gain of protein bands, and altered levels of specific proteins.

The most frequent observation has been altered electrophoretic mobility (e.g. Cooper *et al.*, 1986; Karp *et al.*, 1987; Sabir *et al.*, 1992). In these instances a new band replaced a parental band of relatively equal intensity. This observation suggests alterations in one or a few base-pairs, resulting in an altered enzyme charge or folding structure. It is unlikely that relatively larger insertions or deletions result in a stable, correctly translated protein, especially in the case of isozymes where activity is required in the assay. Dennis *et al.* (1987) and Brettell *et al.* (1986) have provided arguably the most detailed assessment of tissue-culture-induced mutation resulting in altered gene function. In these studies, altered alcohol dehydrogenase 1 mobility in two independent regenerant-derived maize lines was found to be due to different A to T transversions in the coding region of the gene.

Duplications and deletions of bands have been observed. Davies *et al.* (1986) found that change in copy number of alcohol dehydrogenase 1 genes in three independently derived wheat lines was shown to be due to gross chromosomal rearrangements. Few, if any, reports exist of duplication or deletion of individual sequences resulting in altered protein patterns. Small insertions, deletions, and rearrangements have been shown in the genomes of mitochondria and plastids after passage through culture (Shimron-Arbarbanell and Breiman, 1991).

Altered protein expression levels have been explained by different mechanisms. Sabir *et al.* (1992) characterized an altered glucose phosphate isomerase pattern in a sugarbeet regenerant which showed all parental bands but at different levels of activity. This alteration was suggested to result from either a sequence change or a mutation in a *trans*-acting factor. Ryan and Scowcroft (1987) identified a regenerant-derived line containing five new β-amylase bands. This banding pattern was not seen in any of 111 diverse wheat lines. The authors suggested that the new banding pattern was due to activation of a quiescent locus.

Lessons from Documentation of Culture-induced Variation

Literature documenting types of culture-induced variation is extensive, and the preceding summary has reviewed only a small portion of these studies. However, several generalizations can be made which will provide a basis for the ensuing discussion of mutagenesis mechanisms. First, there is little doubt that tissue culture is a mutagenic environment, with observed mutation rates well exceeding those in seed-derived plants. The types of variation encompass a wide spectrum, and appear to be dependent upon the duration of the culture period. However, certain trends emerge. (1) Chromosome rearrangements occur much more frequently than changes in chromosome number. (2) A predominance of the muta-

tions, not due to gross chromosomal abnormalities, could be explained by single base changes. This statement is based on the fact that most mutations are recessive, most restriction fragment length polymorphisms are changes in mobility rather than loss or gain of DNA, and most protein variants are also due to altered mobility or activity rather than gain or loss of function. (3) The transient, non-heritable phenotypes of some primary regenerants, the apparent activation of quiescent loci in some instances, and the instability of a portion of the mutations suggest that epigenetic changes must also be considered as a basis of variation.

Mechanisms of Mutation

With the wide array of mutation occurring in tissue culture, it seems at times difficult to conceive of a pathway that would explain the mutagenesis. To anticipate what underlying pathways might be involved in the mutagenesis, it is first necessary to determine which mechanisms are active in culture and assess their relative importance in determining plant phenotype. Variation-producing mechanisms will be discussed in the context of tissue culture-induced change. Since there are only a few studies which relate an altered phenotype to a molecular change, inferences will be made based on phenotypic characterization described above. Potential underlying pathways will then be discussed, based on the frequency of the various mutagenic processes.

Single Base-pair Change

Single base-pair changes can result from polymerase infidelity during DNA replication, ineffective mismatch repair systems, DNA damage by light or oxidizing molecules, or deamination of methylated cytosine residues. Replication or repair problems would result in both transitions and transversions, light damage (*e.g.* UV light) can be most reactive on thymine dimers, and deamination of methylated cytosines results in C to T transitions.

Relatively few sequence data are available describing tissue culture-induced changes. Two sequenced maize Adhl mutations were due to A to T transversion mutations (Dennis *et al.*, 1987; Brettell *et al.*, 1986). Analyses of DNA based on restriction enzyme digestion and proteins based on electrophoretic mobility indicate that many changes may be small sequence changes. These analyses most often detect DNA or protein bands of altered mobility, rather than loss or gain of bands. Single base-pair changes could reasonably explain a large proportion of tissue culture-induced variation.

DNA Methylation Change

DNA methylation has been associated with gene expression in numerous plant and animal species (Hepburn *et al.*, 1987; Holliday, 1987; Cedar, 1988). The direct role of DNA methylation in gene expression is still a subject of debate,

although the correlation of cytosine methylation and gene expression in mammalian and plant systems is quite good. Increased methylation may reduce gene expression by altering chromatin structure (Klaas and Amasino, 1989) or by direct interaction with proteins which block transcription factors (Boyes and Bird, 1991). Putative activation of quiescent genes in culture has been suggested (Peschke *et al.*, 1991; Ryan and Scowcroft, 1987); reduction in cytosine methylation is one mechanism by which expression of quiescent genes is possible.

Methylation patterns are clearly altered by the culture process (Brown, 1989; Brown *et al.*, 1990; Kaeppler and Phillips, 1993b; Smulders *et al.*, 1995). These methylation changes can be sexually transmitted to regenerant-derived offspring (Kaeppler and Phillips, 1993). However, a direct effect of methylation change induced by culture on gene expression has never been proven.

At least one interesting mutation suggests that epigenetic change may be the basis of at least some tissue culture-induced mutations. Oono (1985) identified a dwarf mutant in rice which was 'homozygous' in the regenerated plant, and stably inherited upon selfing. The mutant could never be recovered upon outcrossing, and normal phenotype was restored by treatment with 5-azacytidine, a chemical known to reduce methylation. Our studies on the stability of tissue culture-induced methylation changes indicate that while alterations are quite stably transmitted through selfing generations, they are much more likely to change upon outcrossing (Kaeppler, 1992).

Several studies of tissue culture-induced change in gene expression indicate that methylation is at least correlated with observed mutations. Transposable elements, detected in regenerant-derived progenies of maize, have been shown in some cases to have reduced methylation (Peschke *et al.*, 1987). Tissue culture of a suppressed Ac element leads to increased expression in regenerant-derived progeny accompanied by decreased methylation (Brettell and Dennis, 1991). Preliminary analysis of five independently derived P-locus (white cob) mutants in maize indicates a substantial increase in methylation in an analysis of regenerant-derived progeny (unpublished data). In all of these examples, methylation status correlates with gene expression but has not been proven as the causal factor. An objective assessment of the potential role of methylation in tissue culture-induced variation would be that methylation change occurs frequently enough to cause many changes in gene expression, but proof of its direct role is not available.

Transposable Elements

Transposable elements can change gene expression in several ways. Insertion of the element into a gene can alter transcription and/or translation of the gene product. Imprecise excision of the element from a gene can leave 'footprints', additions or deletions of a few base-pairs. In some cases, chromosome breakage can occur at the site of a transposable element. Transposable element-induced mutations are often characterized by an unstable phenotype resulting in sectoring of variant and normal tissue.

Active transposable elements have been detected in progeny of regenerant-derived maize plants (Peschke *et al.*, 1987; Peschke and Phillips, 1991). Transposable element activity was detected by outcrossing to a transposable element tester stock; the regenerant-derived lines themselves showed no qualitative or unstable mutations. Few reports of unstable mutations or sectoring of mutated sectors in regenerant-derived progenies have been reported. An unstable flower colour mutation identified by Groose and Bingham (1986) acts like a transposable element-induced mutation, but this hypothesis has not yet been proven. It is our opinion that transposable elements probably account for a relatively small proportion of tissue culture-induced variation.

Small Insertions and Deletions

Small insertions and deletions (e.g. less than 100 kb), not due to transposable element activity, can result from intrageneic or intergenic recombination in a limited chromosome region, integration of small pieces of DNA, or severe problems during DNA replication. The result of small deletions can be loss of a specific gene or gene activity while surrounding genes are retained. Small insertions can result in gene duplication or gene rearrangement.

Little evidence exists for small deletions or insertions in tissue cultures or regenerated plants. As described previously, most studies involving restriction fragment length polymorphisms or protein banding patterns indicate that changed mobility, rather than loss or gain of sequence or function, is responsible. In cases where loss or gain of sequence of function is observed, another explanation such as chromosomal aberrations (e.g. translocations or deletions of chromosome arms) appears most plausible. Small insertions or deletions probably play little role in tissue culture-induced mutation.

Amplification and Diminution of Repeated Sequences

Repeated sequences are present throughout plant and animal genomes. In some cases the function of these sequences is clear, such as dispersed repeats which serve as DNA replication origins. In other cases the exact function of repeats is not known. Heterochromatic blocks of repeated sequences have been shown to exert *cis*-acting position effects. A classic example of this phenomenon is position effect variegation (Eissenberg, 1989). In *Drosophila*, the white eye colour locus exhibits a variegated phenotype when adjacent to a block of heterochromatin, but has the normal phenotype when an inversion ($In(1)w^{m4}$) moves the locus away from the heterochromatic block.

Evidence exists for changes in repeated sequence copy number in tissue cultures, regenerated plants and their progenies. Kidwell and Osborn (1993) assessed repeated sequence variation among alfalfa regenerants. In this study, tandemly repeated sequences were more variable than disperse repeats. Brettell *et al.* (1986) characterized changes at the NOR locus of regenerant-derived triticale plants, and showed that a culture-induced copy number decrease in one regenerant correlated

with an altered C-banding pattern. C-banding was also used to detect change in the position and size of heterochromatic blocks of alfalfa regenerants (Masoud *et al.*, 1991). Total genome size was shown to vary in cultures and regenerants of carrot (Arnholt- Schmitt, 1995).

Changes in sequence copy number would most likely occur in one of two ways. (1) Chromosome breakage events could result in loss of chromosome arms, or deletions or duplications of chromosome regions by breakage followed by reunion of broken ends. Chromosomal aberrations resulting from chromosome breakage have been found to occur frequently in culture (Lee and Phillips, 1988), and have been shown to explain aberrant isozyme patterns in at least one study (Davies *et al.*, 1986). (2) Changes in copy number could result by unequal cross-over events. Recombination among homologues in culture has been suggested by some authors (Roth *et al.*, 1989), but clearly not proven. Our limited analysis of hybrid regenerants with RFLP markers has always shown both parental alleles indicating that, at least in maize, somatic recombination among homologues is not common (unpublished data). Sister chromatid exchange (SCE) frequencies have been shown to increase under conditions of cellular stress in mammals, and increased rates of SCE have been detected in cultured plant cells (Dolezel and Novak, 1986; Dimitrov, 1987).

Repeated sequence copy number change probably does occur in cultured cells due to mechanisms other than chromosome breakage. The effect of blocks of heterochromatin on gene expression is poorly understood in non-culture systems, making it difficult to predict how repeat copy number changes induced by culture might translate to an altered phenotype. Furthermore, the frequency of such events is not well characterized. Change in chromosome and chromatin structure due to alteration in the copy number of repeats appears to be a 'wild-card' in our understanding of culture-induced variation.

Gross Chromosome Rearrangements

Chromosomal aberrations such as translocations, inversions, and deletions can alter gene expression by eliminating certain sequences, moving genes to another part of the genome, or direct gene interruption if the breakpoint falls in the coding region itself. Changes in chromosome number such as aneuploidy, polyploidy, or haploidy can result in phenotypic change due to increase or decrease in some or all of the genes in a plant. For example, changing maize from the diploid to the tetraploid level usually results in a larger plant with larger cells and anatomy; monosomy in maize can result in smaller and less vigorous plants.

Karyotypic changes are relatively frequent and have the potential to affect the phenotype of cultured cells and primary regenerants. However, many of the karyotype changes having the most phenotypic effect (e.g. deletions, aneuploidy) may not be readily transferred to regenerant-derived progeny, especially in diploid species. Therefore, cytological aberrations may be most important in explaining gene expression changes in primary regenerants, but of less import-

ance when seed-derived progenies are the final outcome of a tissue culture experiment.

Potential Underlying Pathways

The mutagenic nature of tissue culture is now evident. The looming question then remains, 'Why does tissue culture change the genome?'. Tissue culture has been suggested as an environment which is stressful to cells (McClintock, 1984). If so, what is the stressor, when all nutrients and metabolites are apparently provided? Tissue culture mutation has been described as an extreme attempt by the plant cell to adapt to an imperfect and altered environment. Tissue culture was developed through an understanding of the wound response in plants. Perhaps the wound response prepares cells to produce a terminal callus structure, not to regenerate to plants.

While these philosophical types of discussions mostly illustrate how far we are from understanding tissue culture-induced variation, it is important to proceed with a hypothesis as to why the cells respond in the manner they do. Our hypothesis in the proceeding discussion will be that tissue culture is a stress environment, and that the cellular response is an attempt to survive rather than an apoptotic mechanism. Furthermore, the discussion will proceed with the following generalizations and inferences in mind. They are made based on experience and based on review of a large literature, but are probably not documented in any single paper.

1. The frequency of observed tissue culture-variation is age-dependent. Cells in culture accumulate mutation over time and there is no evidence that the mutation rate differs over time.
2. Tissue culture-induced variation is genotype dependent. In general, it appears that, on a per generation basis, the genotypes which respond best to culture and grow most rapidly incur less mutation. Anecdotal evidence also suggests that selective agents in the culture, such as antibiotics or herbicides used in transformation experiments, may enhance variation.
3. The most frequent mutations occurring in culture are chromosome aberrations due to chromosome breakage events, DNA methylation changes, and sequence change which we will presume at this point to be single base changes. The chromosome breakage events are related to heterochromatin, probably due to incomplete replication before the S to G_2 transition.
4. The health of many cultures reduces with age, resulting in decreased regeneration ability and reduction in growth rates. Some cell lines seem to have been 'immortalized' and propagated for 10 or more years. These cell lines, without exception, will not regenerate plants.

Based on these suppositions, a series of hyopotheses on potential underlying pathways will be reviewed or put forth. It is hoped that a summary of these ideas will

stimulate thinking. However, the ideas are based primarily on speculation, and interrelationships of the mechanisms are not clear.

Hypothesis: Late Replication of Heterochromatin is Due to a Nucleotide Pool Imbalance

Chromosome breakage is clearly a frequent event in tissue culture, and breakage appears to be involved with heterochromatin. Lima-de-Faria (1969) has shown that heterochromatin replicates late in the cell cycle. Late replication of heterochromatic knobs in maize has been found in certain genotypes of maize, resulting in anaphase bridges and breaks between the knobs and the centromere. Interestingly, the primary location of breaks induced by tissue culture is between heterochromatic knobs and the centromere. This observation led to a unifying hypothesis implicating chromosome breakage as the primary event in tissue culture variation (Lee and Phillips, 1988). This hypothesis was later extended to explain the various types of mutation that were subsequently characterized (Phillips *et al.*, 1990, 1994; Peschke and Phillips, 1992; Kaeppler and Phillips, 1993a).

A critical question in this hypothesis is why heterochromatin apparently fails to complete replication before the S to G_2 transition. The S to G_2 and G_2 to M transition in many cell types is usually tightly regulated. It is not normal for mitosis to proceed before replication has completed. The cell cycle of culture cells is generally longer than that in root-tip meristems, where cell division proceeds without damage. Gould (1984) has shown that the primary difference in cell cycle length is due to the G_1 phase which has a duration of 23.1 h in cultured cells versus 1.7 h in root-tip meristems of maize.

Among the possible explanations for incomplete replication of heterochromatic regions is a nucleotide imbalance in the cell culture. Heterochromatin is usually A-T rich, so a relative lack of a critical nucleotide, such as adenine, could result in replication difficulties. Furthermore, simply the ratio of the components rather than molar concentration may be of even greater importance. Most tissue culture media were formulated by empirical results, rather than based on precisely quantified requirements.

While numerous media component levels could reasonably be tested, the complexity of most media formulations often leads to a nearly unmanageable multifactorial experiment to assess optimum component levels. Furthermore, it is not a straightforward proposition to assess tissue culture stability. Mutation is measured in terms of frequency rather than absolute presence or absence. Assessments are usually based on regenerated plants rather than individual cultured cells. Regenerated plants probably reflect only a subset of the variants present in the progenitor culture itself. Should nutrient levels in the media be the critical factor in inducing chromosome breakage, there is little hope that the problem can be resolved by trial and error. Rather an increased understanding of cell division and DNA repair in plant cells in general will hopefully narrow studies of critical media components and metabolites which may be involved in the mutagenesis pathway.

A second hypothesis for heterochromatin-induced breakage involves DNA methylation and altered chromatin structure. Knob heterochromatin in maize is highly methylated and heterochromatic. If these sequences become even more methylated and heterochromatic in culture, the result may be non-disjunction during mitosis. The non-disjunction in this case would be due to DNA packaging, rather than incomplete replication. Perhaps molecular studies on B-chromosome-induced chromosome breakage involving knobs (Rhoades and Dempsey, 1972, 1973) would shed light on chromosome behaviour in tissue culture.

Hypothesis: Methylation Alterations Affect DNA Repair Processes Leading to Single Base-pair Changes

Cytosine methylation change in cell culture occurs very frequently relative to seed-derived plants. Methylation has been correlated with gene expression, and chromatin structure. The high frequency of methylation change coupled with the important gene expression processes with which it is associated, suggests that methylation change may be involved in tissue culture-induced mutagenesis.

One hypothesis would be that a biochemical or signal transduction pathway results in methylation changes, changes which ultimately lead to sequence and chromosome alterations. The mechanism of methylation change has not yet been characterized. The most likely possibilities are (1) maintenance or *de novo* methylation activities are directly altered by the culture process, or (2) methylation alterations reflect gene expression changes, mediated by DNA binding proteins affecting maintenance methylation.

Recent results in mammalian systems indicate that expression of the DNA methyltransferase plays a critical role in signal transduction and transformation of cultured mammalian cells from anchorage-dependent to anchorage-independent. Increased activity of the DNA methyltransferase has been correlated with the state of malignancy of the cell (El-Deiry *et al.*, 1990). Expression of the mammalian methyltransferase has been shown to be under the control of the Ras-Jun signal transduction pathway (Rouleau *et al.*, 1995). This pathway is important in controlling cell division. MacLeod and Szyf (1995) found that reduction in methylation of transformed lines via an antisense methyltransferase or by treatment with methyltransferase inhibitors caused reversion of the transformed phenotype. Generally speaking, transformed mammalian cells are more genetically stable than non-transformed cell lines and do not show an age-dependent reduction in viability.

Methylation change in plant tissue culture is rather complex. Single copy sequences show primarily decreases in methylation, whereas the methylation across the genome is increasing based on HPLC analysis of percentage methylated cytosines (Olhoft, 1996). Based on this observation, an analogy to the animal model is not clear. However, direct activity of DNA methyltransferases may be important in plant cell cultures, as has been shown in mammalian cells.

DNA methylation may play a role in the frequency of single base-pair changes in culture. Phillips *et al.* (1994) have discussed the similarities between tissue

culture-induced variation in plants and a repeat-induced point mutation mechanism in *Neurospora.* An alternative hypothesis, based on DNA repair, is that decreased methylation reduces the ability of methyl-directed DNA repair systems to correctly repair DNA synthesis errors based on the parental DNA strand.

Repeat-induced point mutation (RIP) was first described in *Neurospora* by Selker and Stevens (1985). In brief, sequences inserted into the *Neurospora* genome that have homology to endogenous sequences cause both sequences to become methylated and to diverge by methylation-related C to T transitions. By this mechanism, methylated cytosines are deaminated and C to T transitions occur in the mismatch repair process. The attractive similarities between RIP and tissue culture-induced variation are the high frequency of methylation and sequence change. Incongruities in the hypothesis include the fact that many reported methylation changes are decreases in methylation (Kaeppler and Phillips, 1993b), and the fact that duplicated sequences in maize are apparently stable in seed-derived plants.

Methyl-directed mismatch repair is a process characterized in *E. coli* (Modrich, 1994). The existence of a methyl-directed mismatch repair system in higher plants has not yet been shown. By this process, mismatched base-pairs are repaired to the correct base using methylation of the parent strand as a marker to determine which DNA strand was altered during replication. Lack of DNA methylation within a genomic region would remove the ability of this system to detect the parental DNA strand. Reduced efficiency of this system is consistent with the changes induced by culture since changes in single copy regions are primarily hypomethylation events (Kaeppler and Phillips, 1993b; Olhoft, 1996).

Several testable hypotheses can be made in comparing the RIP versus methyl-directed repair models to explain single base changes. The RIP model requires that (1) methylation of mutated sequences increases in culture, (2) changes are primarily C to T transitions due to deamination of methylated cytosines, and (3) sequences which most frequently mutate are duplicated, and show the most methylation increases. The methyl-directed mismatch repair model requires that (1) methylation of mutated sequences decreases in culture, (2) transitions and transversions occur at equal frequencies, and (3) sequences showing the most hypomethylation also show the most mutation. Research to compare the potential occurrence of each type of event by determining the type of mutation would be extremely useful. A preponderance of C to T changes would support a RIP-like mechanism. Equal transition and transversion frequency would support a model based on a decrease in the efficiency of methyl-directed mismatch repair due to a decrease in methylation.

Hypothesis: Byproducts of Cell Death and Degradation Elicit a Mutagenic Response

Two observations, which have not yet been appropriately documented, open an interesting line of thinking as to potential elicitors of the mutagenic response. First, genotypes which grow best in culture seem to have lower mutation frequen-

cies, especially on a per-cell generation basis. Second, toxic agents, such as antibiotics or herbicides used in DNA transfer experiments, may result in higher rates of variation in the resistant, transformed cells.

Stressed and dying cells clearly produce secondary metabolites such as pigments and tannins, and probably release compounds into the media coincident with cell death and degradation. Genotypes which respond poorly to culture usually have a higher proportion of stressed or dying cells, and selection of transgenic colonies is certainly done in a culture environment where a majority of the cells are dying.

Perhaps cells under severe stress can elicit a response in surrounding healthy cells, release cytotoxic compounds, or cause nutrient stress in healthy cells by affecting transport of nutrients to cells around them. Several types of experiments could address this possibility. Extracts of dying cells could be added to media of a culturable genotype to determine whether mutation frequency is affected. Selective compounds which only inhibit cell growth could be compared to those that kill cells relative to the mutation frequency in resulting regenerated plants. Such a mechanism would not only have ramifications for controlling tissue culture-induced variability, but might also be important in determining selective agents which could minimize variability in transformed plants.

Hypothesis: Tissue Culture-induced Variation is Caused by Hormones used in the Culture Process

A critical component of the tissue culture system is hormones which elicit the culture response. With such large effects on the expression of genes in a cell, it seems possible that these hormones could also be responsible for the mutagenic effect of culture.

2,4-dichlorophenoxyacetic acid (2,4-D) is a synthetic auxin commonly used in culture media. This chemical has been shown to be mutagenic in both plant meristematic cells and animal cell cultures resulting in mutation, chromosome breakage, and enhanced rate of sister-chromatid exchange (Dolezel *et al.*, 1987; Pavlica *et al.*, 1991). Cells clearly undergo substantial gene expression changes during the culture initiation process. This de-differentiation process has been shown to be accompanied by a change in overall methylation (LoSchiavo *et al.*, 1989; Vergara *et al.*, 1990; Arnholt-Schmitt *et al.*, 1995) as well as variation in genome size due to repeated sequence amplification and diminution (Arnholt-Schmitt, 1995). These drastic genomic changes may in themselves elicit genome instability and the accompanying phenotypic variability.

Hormones involved in cellular senescence may also be important in tissue culture-induced variation. Plant cell senescence may be considered analogous to programmed cell death in mammals. A reduction in genome stability is probably associated with the senescence process. Ethylene is a hormone important in growth, differentiation, and senescence in plants. The direct importance of ethylene in culture growth is not clear, although ethylene inhibitors have been shown to enhance callus growth and morphology in several species. For example, Vain

et al. (1989) and Songstad *et al.* (1991) have shown the beneficial effects of silver nitrate, an ethylene inhibitor, in promoting Type II (embryogenic) callus production in maize. This type of callus is generally faster-growing and may exhibit less mutation than organogenic callus of the same genotype. Silver nitrate has not shown an effect in all culture systems.

Cytokinins are also important in cellular senescence. Regulation of cytokinin levels in transgenic tobacco by a novel isopentenyl transferase control mechanism has resulted in a marked reduction in the rate of leaf senescence (Gan and Amasino, 1995). Perhaps genome destabilization is a senescence type of mechanism exhibited in cell cultures because of imbalances in specific hormone levels, such as cytokinins.

Hormones are critical components of the tissue culture process. It would be unfortunate if the very chemicals which elicit the culture response also destabilize the genome. Probably the hormonal balance is critical to cell growth and genome stability. Subtle variations in this balance may be the factor which allows cell growth, but also results in genome instability. A more complete understanding of genome stability, tissue senescence, and plant cell division in normal systems will ultimately lead to a better understanding of plant cell growth in culture.

Summary

Plant tissue culture is a widely used procedure, and variation induced by the culture process has been extensively studied. It is frustrating, therefore, to have to summarize a review without an answer to the two most fundamental questions regarding tissue culture-induced mutation: (1) What elicits genome instability?, and (2) Does tissue culture produce genetic variation in a way that is unique among mutagenic agents? To date, we can only cite the myriad of papers documenting culture-induced variation and propose hypotheses which are often difficult to test experimentally.

In spite of the enigmatic nature of this phenomenon, the potential to harness this variation in order to tackle specific problems looks promising. Tissue culture is unique among plant selection systems in that millions of experimental units can be selected in a very small area. In circumstances where alteration in a specific biochemical pathway is desired, selection can be very efficiently accomplished. An example is selection for herbicide resistance, where resistance to a specific chemical is desired. A second example is resistance to feedback inhibition of an enzyme for pathways in which enzyme-inactivating analogues are available. In targeted situations such as these, selection can be done efficiently in a small incubator; the same experiment on field plants would cover many hectares of land.

Variants induced by culture are generally not unique. Chromosome breakage events and epigenetic variants probably occur at a higher frequency than with other mutagens (e.g. ethylmethanesulphonate), but single base changes also occur regularly. Many targeted changes, such as the metabolite analogue selection

scheme discussed above, require single base mutations. Tissue culture provides this type of mutation at a frequency which will allow efficient selection.

While the basis of tissue culture-induced mutation is still poorly understood, useful genotypes have been selected based on this mutagenesis. Understanding the mutation process remains a problem of fundamental importance in plant biology. This understanding will allow elimination of variation in experiments where it is not desired, or enhance variation where it is needed. In addition, basic understanding of plant genome stability will be enhanced. The large amount of information now available from many areas of plant growth, development, and biochemistry will provide the clues to ultimately solving this important problem.

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24. Molecular and Biochemical Characterization of Somaclonal Variation

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Introduction

Somaclonal variation may result from a wide range of genetic changes (Phillips *et al.*, 1994; Karp, 1991, 1995; Jain, 1996). Major chromosomal re-arrangements may occur during tissue culture of plant cells (Orton, 1980b; Lapitan *et al.*, 1984). Chromosomal duplication and increase in ploidy are often reported. At the other extreme, single base or point mutations are a mininal type of genetic change that results in somaclonal variation. Transposable elements may also be the source of variation. Somaclonal variation in nuclear, chloroplast and mitochondrial genomes is possible and changes in more than one of these genomes or by more than one of these mechanisms may be found in any individual somaclone. Several mitochondrial genotypes may exist in equilibrium in plant cells, with the equilibrium being disrupted in cell culture resulting in the appearance of previously rare genotypes (Shirzadegan *et al.*, 1991). Deverno (1995) reviewed somaclonal variation during somatic embryogenesis of woody plants and concluded that somaclonal variation was an important source of new traits for plant improvement especially for this type of plant (trees and other long-lived perenial species). However, somaclonal variation can also introduce unwanted variation in attempts to propagate plants clonally.

Epigenetic (non-genetic) changes may also be associated with somaclonal variation (De Klerk, 1990; Bauman and De Klerk, 1995). Epigenetic changes appear frequently in regenerated plants as a result of physiological responses of the plant cells to the conditions imposed in culture and regeneration. Many of these phenotypic changes are not heritable and may not be displayed in subsequent generations. However, several mechanisms may lead to changes in gene expression that may persist for more than one generation. Methylation of DNA has been associated with altered patterns of gene expression (Doerfler, 1983; Lo Schiavo *et al.*, 1989). Increased methylation of cytosine may result in reduced expression. DNA methylation is replicated in meiosis allowing transmission to the next generation. However, analysis of variation in the total extent of methylation may not explain the changes in the expression of individual genes which may be differentially methylated.

Somaclonal variation may be analysed by the study of phenotypic variation or by investigation of underlying genetic variation. Molecular and biochemical analysis of somaclonal variation provides an opportunity to investigate the extent and nature of somaclonal variation and to provide a quantitative measure of somaclonal variation. This analysis may be useful in both the minimization of

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somaclonal variation (e.g. in plant transformation and commercial plant propagation – Chatterjee and Prakash, 1993) and in the exploitation of this source of genetic variation (e.g. in crop improvement – Adkins *et al.*, 1995). The genetic variation in microspore-derived doubled haploids (and its molecular characterization) has recently been reviewed (Logue, 1996). This may be considered as gametoclonal variation, as distinct from somaclonal variation, and some different mechanisms may be involved. However, the methods available for biochemical and molecular characterization are essentially the same. Molecular analysis may also help to distinguish genetic and epigenetic components of somaclonal variation.

Different molecular and biochemical techniques can be employed to detect the full spectrum of somaclonal variation that may arise by mechanisms that range from chromosome rearrangement or breakage (Lee and Phillips, 1988) and activation of transposable elements (Peschke *et al.*, 1987) to point mutations.

Molecular Methods Available For Use in the Characterization of Somaclonal Variation

A wide variety of methods are available to characterize plant genomes. These methods include:

1. Restriction fragment length polymorphism (RFLP).
2. Random amplified polymorphic DNA (RAPD).
3. DNA amplification fingerprinting (DAF).
4. Amplified fragment length polymorphism (AFLP).
5. Short sequence repeats (microsatellites) (SSR).
6. Temperature gradient gel electrophoresis (TGGE).
7. Denaturing gradient gel electrophoresis (DGGE).

Many of these methods have potential for application in the analysis of somaclonal variation. However, only RFLP and RAPD analysis have been applied widely in analysis of somaclonal variation (Table 1). The most appropriate method for any particular application is likely to depend upon factors such as the extent and nature of variation, the background knowledge of the genome of the species being studied, the availability of sequence data or DNA probes and the availability of plant material and laboratory facilities.

A wide range of techniques may often be applied to the same system. For example, Wolff *et al.* (1995) used RAPD, SSR, and RFLP markers to evaluate somaclonal variation in vegetatively propagated chrysanthemum cultivars.

Specific genetic lines such as the mutator lines of maize may provide a unique opportunity to study somaclonal variations at the molecular level (James and Stadler, 1989).

The molecular basis of somaclonal variation will be described elsewhere in this volume. The analysis of somaclonal variation may be targeted to detect specific types of variation. different types of analysis are more appropriate for the detec-

Table 1. Some examples of biochemical and molecular analysis of somaclonal variation

Species	Material analysed	Technique	Variation observed	Reference
Maize	Callus	RFLP	Yes	Brown *et al.*, 1991
(*Zea mays*)	Regenerated plants	nuclear	Yes	Gengenbach *et al.*, 1981
		Mitochondrial		McNay *et al.*, 1984
Sugarcane	Callus	RAPD	Yes	Taylor *et al.*, 1995
Beta vulgaris			No	Oropeza *et al.*, 1995
Beet	Regenerated plants	RFLP	Yes (0.05–0.1%)	Sabir *et al.*, 1992
Beta vulgaris		Isozymes		
Rice	Suspension cultures	RFLP	Yes	Saleh *et al.*, 1990
(*Oryza sativa L.*)	Regenerated plants	mitochondrial		Muller *et al.*, 1990
		Chloroplast		Harada *et al.*, 1991
				Kawata *et al.*, 1995
Fescue	Regenerated plants	RFLP	No	Valles *et al.*, 1993
(*Festuca pratensis Huds*)		RAPD		
Brassica campestris L.		RFLP	Yes	Shirzadegan *et al.*, 1991
	Callus culture	mitochondrial		
Hordeum marinum		RFLP	Yes	Shimron-Abarbanell and Breiman, 1991
	Regenerated plants	nuclear		
		Mitochondrial		
		chloroplast		
Triticale	Regenerated plants	RFLP	Yes	Brettel *et al.*, 1986

tion of variation at different levels (e.g. point mutations or chromosomal rearrangements) and in different genomes (e.g. nuclear or mitochondrial).

RFLP Analysis

Restriction fragment length polymorphism (RFLP) analysis was the first widely used method for analysis of differences in plant DNA. During the past decade, RFLP maps have become available for many plant species (Paterson *et al.*, 1991). Large numbers of RFLP probes are available from these mapping studies, allowing the selection of sets of probes to scan the genome for genetic changes. RFLP markers offer great potential to analyse plant genomes for somaclonal variation and have been used successfully to study somaclonal variation. Genomic or cDNA probes from the species of interest or related species are used to detect polymorphisms in the length of restriction fragments in genomic digests. This approach allows the detection of even single base mutations at the restriction site. Recent advances allow the use of non-radioactive labelling of DNA probes. This results in a stable labelled probe that can be stored and used over time to analyse different samples. The main limitation of this technique is that a wide selection of polymorphic probes are not readily available for all plant species. Well-mapped species such as the major crops are readily amenable to RFLP analysis but more obscure species may require investment of considerable development effort to isolate and characterize suitable RFLP probes. Methods based upon the polymerase chain reaction (PCR) are also more likely to be favoured because of simplicity, speed, and in some cases cost. Methylation-sensitive restriction enzymes may be used to study changes in methylation (Arnholdt–Schmitt *et al.*, 1995).

RAPD Analysis

Random amplified polymorphic DNA (RAPD) is another method that allows scanning of the genome for genetic changes (Williams *et al.*, 1990). The method uses short oligonucleotide primers of arbitrary sequence in a polymerase chain reaction PCR to generate molecular markers. The markers can be easily analysed by electrophoresis on agarose gels with ethidium bromide staining. Several variations have been employed to enhance the method, including restriction digestion of the products and analysis on polyacrylamide gels with silver staining. However, for most purposes the simpler approaches are adequate to reveal useful polymorphisms. The advantages of RAPD compared to alternative methods include the simplicity of the technique and the lack of a requirement for suitable probes or for prior knowledge of sequences in the species being analysed, the speed of analysis and the relatively large number of genetic loci that can be analysed. This has resulted in RAPD becoming a favoured method for analysis of somaclonal variation. RAPD analysis is difficult to reproduce, especially between laboratories, and is probably best used to compare within a set of samples analysed in the same batch using identical reagents, equipment and analysis protocols. Results can be

very reliable and informative when the technique is carefully applied. A large amount of data can be collected easily, allowing analysis of very small genetic differences that cannot be easily uncovered with alternative techniques. Each oligonucleotide primer results in the amplification on average of up to about 10 scorable genetic markers, and large numbers of primers are available for screening. Comparison of individuals at several hundred genetic loci is thus readily achieved. DNA amplification fingerprinting (DAF) is a slightly different protocol for analysis with primers of arbitrary sequence (Caetano-Anolles *et al.*, 1992) and might prove useful in analysis of somaclonal variation in some systems.

AFLP Analysis

Amplified fragment length polymorphism (AFLP) is a technique with the potential to analyse genetic variation intensely. The method involves digestion of genomic DNA with restriction enzymes, ligation of linkers and selective PCR amplification with primers complementary to the linkers but with unique 3′ overhangs (Zabeau and Vos, 1993). This method generates very large numbers of markers requiring a polyacrylamide gel similar to that used for DNA sequencing. The large number of markers detected with this method provides good sensitivity for the detection of somaclonal variation. Very large numbers of AFLP markers have been screened in some plant species (Thomas *et al.*, 1995).

SSR Analysis

Microsatellites or short sequence repeats (SSR) detect highly polymorphic loci and may be useful in the characterization of somaclonal variants. The number of repeats of the di- tri- or tetra-nucleotide unit is highly variable between individuals in many populations (Tautz, 1989). PCR amplification of the microsatellite using primers to unique sequence, flanking the repeat region, results in fragments with variable lengths. Microsatellites can distinguish large numbers of alleles but require considerable effort to isolate and characterize. Microsatellites have a high polymorphism information content (PIC) because of the large number of polymorphic alleles, and multiplexing in analysis of microsatellites is possible (Rodder *et al.*, 1995). Microsatellites are generally species-specific so that cloning and sequencing of microsatellite loci must be conducted for each species to identify suitable primers for their detection. Microsatellites may be of comparatively little value in the analysis of somaclonal variation as they each target the analysis of variation at a single specific locus while techniques such as RAPD and AFLP can 'scan' the genome and test for variation at large numbers of loci.

Rus-Kortekaas *et al.* (1994) compared microsatellites and RAPD markers for the analysis of genetic variation in tomatoes and found higher frequency polymorphism using the microsatellites. SSR and RAPD analyses gave results both supporting the same characterization of homozygous potato lines derived from anther-culture (Veilleux *et al.*, 1995).

DGGE Analysis

Denaturing gradient gel electrophoresis (DGGE) can be used to detect polymorphisms in genomic DNA digests (Riedel *et al.*, 1990). This technique is an alternative to RFLP analysis.

Specific PCR-based Methods

Specific PCR-based methods may be applied to the analysis of genetic variation at any locus for which sequence information is available. A wide range of such methods are available, especially in commonly studied species. Heteroduplex analysis using temperature gradient gel electrophoresis (TGGE) is a special case that can reveal sequence differences in the target gene (Tsuchiya *et al.*, 1994). Ribosomal genes are universal and show some somaclonal variation (Brown and Lorz, 1986). Techniques for the analysis of ribosomal genes by PCR are applicable to all plant species (Ko *et al.*, 1994) and provide a opportunity to test gross somaclonal variation. Brettell *et al.* (1986) found that the Nor locus (tandem repeats of ribosomal RNA genes) in triticale was relatively stable in tissue culture, but RFLP analysis revealed a reduction in the number of rRNA units in one line. PCR can be applied to samples as small as a single protoplast (Brown *et al.*, 1993).

DNA Sequencing

The widespread availability of automated DNA sequencing makes this an option for comparing somaclonal variants at specific loci. Comparison of the sequences of specific genes in individual somaclonal variants could be targeted at the analysis of genes or genome regions known to display high somatic polymorphism. The simplicity and cost of DNA sequencing continues to improve, and sequencing is likely to be an increasingly attractive option for molecular analysis of somaclonal variation. The direct cycle sequencing of PCR products has eliminated the need to clone DNA fragments prior to sequencing. This technique allows the direct sequencing of the same locus amplified from different somaclonal lines by PCR.

DNA Isolation for Analysis of Somaclonal Variation

All of the above methods require the isolation of DNA from the plant material. The different methods may require different amounts or purities of DNA. However, if large numbers of somaclonal lines are to be screened, simple and efficient methods for DNA preparation must be employed. Several recent methods offer simplicity and are suitable for analysis of relatively small amounts of plant tissues, ensuring that sampling need not be destructive of the viability of the plant being analysed. The method of Thomson and Henry (1995) requires only milligram quantities of tissue and involves briefly heating the tissue in a solution and

then using an aliquot of the solution (usually diluted) directly in PCR. RAPD analysis of some tissues may require more conventional protocols (Graham *et al.*, 1994) because of the sensitivity of PCR with short arbitrary primers to variations in template concentration and the presence of variable amounts of PCR inhibitors.

Analysis of Marker Genes

The genetic transformation of plants often involves the introduction of foreign genes into plant cell cultures. The requirement for a cell or tissue culture stage provides an opportunity for the introduction of somaclonal variation. The objective is often to introduce a single additional gene into an elite variety without introducing any other changes. Molecular analysis can be used to assess alternative transformation strategies so as to determine the protocol that introduces the least risk of somaclonal variation. Techniques such as RAPD analysis allow the evaluation of genetic variation at multiple loci giving an overall assessment of genetic differences.

Molecular analysis of marker genes in transgenic plants maintained in tissue culture has been used to study somaclonal variation. Somaclonal variation in tobacco and *Arabidopsis* was investigated by analysis of recombination of β-glucuronidase as a non-selectable marker (Puchta *et al.*, 1995). This approach has the potential to allow the tracking of somaclonal rearrangements within the plant genome, leading to an improved understanding of the mechanisms of somaclonal variation. Analysis of tobacco plants showed that the frequencies of recombination were additive for allelic transgenes and increased in the presence of DNA-damaging agents (Table 2).

Some Examples of Molecular Analysis of Somaclonal Variation

Some examples of the results of applying the above techniques to the analysis of somaclonal variation will now be described. Molecular analysis can be applied to the study of nuclear, mitochondrial and chloroplast genomes (Jack *et al.*, 1995).

Table 2. Recombination events detected in transgenic tobacco plants using beta-glucuronidase as a marker gene (Puchta *et al.*, 1995). Effects of allelic state and the presence of DNA-damaging agents

Events/plant	Number of recombination events	Number of plants	Relative number
Hemizygous	242	124	0.5
Homozygous	899	214	1.0
MSS treated	770	100	1.8
Exposed to UV	308	30	2.5

Analysis of Nuclear Genome

RFLP

RFLP analysis of plants regenerated from tissue culture has been used to show that the level of DNA polymorphism increases with the duration of time in culture (Muller *et al.*, 1990). In a study of maize plants regenerated from callus cultures, Brown *et al.* (1991) found that RFLP differences included changes in the number of gene copies. RFLP analysis is probably able to detect a wide range of somaclonal variations from point mutations (at the restriction site) to large chromosomal rearrangements (resulting in changes in the distance between restriction sites). Arnholdt-Schmitt (1993) applied RFLP analysis to the study of repetitive sequences in carrot and found changes in copy number during callus induction and plant regeneration. Similar results were reported for musk melon (Grisvard *et al.*, 1990). Brown *et al.* (1990) concluded that RFLP polymorphisms were widespread and not associated with specific genomic regions in rice plants derived from protoplasts.

RAPD

Some recent studies illustrate the use of RAPD analysis to evaluate somaclonal variation. Rani *et al.* (1995) identified somaclonal variants of *Populus deltoides* using RAPD markers. Six of 23 plants propagated from a single clone could be distinguished from the others and field-grown plants by 13 polymorphisms using five primers. This result suggests that a single somatic mutation event has been detected that preceded the cell division that resulted in the cells contributing to the six identical variants.

Taylor *et al.* (1995a) applied RAPD analysis to evaluate callus derived from sugarcane protoplasts and plants regenerated from embryogenic callus as well as transgenic sugarcane plants. RAPD analysis was able to detect genetic differences in the protoplast-derived callus that had been subjected to prolonged culture. These cells had been in culture for more than 2 years and had lost the ability to regenerate into plants. No genetic differences could be detected in transgenic plants, although some plants displayed morphological differences that were probably largely epigenetic. Analysis of PCR with 41 primers gave 204 scorable markers (an average of 12 markers per primer; Taylor *et al.*, 1995b). The markers averaged 1.1 kb and at 12 per primer sampled an average of 13.2 kb. This represents only 0.0001% of the genome (1.2×107 kb (2C) in sugarcane. A very large number of RAPD primers would be needed to sample the genome for minor changes.

Somaclonal variants resistant to sugarcane mosaic virus (SCMV) were distinguished from susceptible genotypes using RAPD markers (Oropeza *et al.*, 1995). The molecular markers were shown to be stable and linked to the resistant genotypes over seven generations of field-grown plants, demonstrating their reproducibility and stability.

AFLP

AFLP analysis is expected to provide a very valuable tool for the analysis of somaclonal variation because of the very large number of markers that can be generated. Analysis of transgenic plants with AFLP markers has revealed polymorphisms relative to the parental line apparently associated with somaclonal variation (B. Carrol, personal communication).

Analysis of Chloroplast Genome

RFLP

Chloroplast genomes are generally considered to be more highly conserved and more stable than mitochondrial genomes, and less likely to undergo extensive somaclonal variation. However, major somaclonal changes in chloroplast genomes have been reported. Analysis of the chloroplast genome of rice cultures indicated that prolonged culture resulted in the deletion of parts of the genome (Kawata *et al.*, 1995). These deletions were associated with changes in plastid morphology. Large deletions of chloroplast DNA were often associated with albino plants regenerated from anther culture or tissue culture (Harada *et al.*, 1991). The rice plastid deletions studied by Kawata *et al.* (1995) resulted in the retention of a region between positions 15 000 and 22 000. One end of all deletions (except for one) was found between positions 15 000 and 43 000. This is the region that has undergone significant evolutionary rearrangements in angiosperms.

RAPD

Somaclonal variations resulting in dwarf off-types have been observed among tissue-cultured bananas. RAPD analysis has been use to identify a molecular marker that can be used to screen for off-types in the laboratory (Damasco *et al.*, 1996). A RAPD marker was found to be lacking in all dwarf banana lines from several genotypes and to be present in all normal lines. The marker has great value in the screening of bananas in tissue culture to ensure elimination of dwarf types. The marker was found to be associated with the chloroplast genome. Sequencing of this marker and analysis of the chloroplast genomes of off-type plants may define the exact molecular basis of this high-frequency somaclonal variant.

This type of RAPD marker may be worth converting to a more robust PCR marker a sequence characterized amplified region (SCAR) or sequence tagged site (STS)) by sequencing and designing of longer PCR primers. This approach allows PCR under more stringent conditions, permitting the use of simple and rapid DNA extraction protocols for use in screening large numbers of samples. Elimination of electrophoretic analysis is also possible in such cases. Alternatives allow colorimetric or fluorometric detection (Holland *et al.*, 1991) when the sequence of the PCR product to be detected is known. These developments should

permit routine molecular screening of tissue cultures to allow elimination of high-frequency somaclonal variants at an early stage.

Analysis of Mitochondrial Genome

RFLP

Analysis of *Brassica campestris* callus suggested that the changes in mitochondrial genome in culture resulted from preferential amplification of minor, pre-existing forms that arose by infrequent rearrangements of short dispersed repeated elements (Shirzadegan *et al.*, 1991). Analyses of somaclones from maize suggest that mitochondrial genome changes may arise during culture (Gengenbach *et al.*, 1981) or through changes in the frequency of specific mitochondrial genotypes (McNay *et al.*, 1984). The relative contribution of these two processes should be determined by further molecular analysis of these systems.

Level of DNA Methylation

The level of DNA methylation has often been assessed in studies of somaclonal variation. Muller *et al.* (1990) found a strong correlation between the level of DNA polymorphism (due to rearrangements of the DNA) and the level of methylation in rice. This may result from methylation of altered sequences, or simply because both the changes in DNA sequence and methylation are accelerated in culture. Auxin levels in culture have been reported to relate positively to the level of DNA methylation in carrot cultures (LoSchiavo *et al.*, 1989) as shown in Table 3. The level of methylation of DNA increased during culture of carrot tissue with the presence of IAA and inositol in the medium, increasing the level of methylation (Arnholdt-Schmitt, 1993). However, DNA methylation was lower in carrot tissue exposed to kinetin (Arnholdt-Schmitt *et al.*, 1991). Some of the methylation in tomato callus (Smulders *et al.*, 1995) is detected in regenerated plants, and part of this is passed to progeny of the regenerated plants.

Analysis of maize plants derived from tissue culture using methylation-sensitive restriction enzymes demonstrates changes that both increase and decrease methylation (Kaeppler and Phillips, 1993). This study also found that changes in methylation were homozygous, suggesting a non-random process. Demethylation may be a significant contributor to somaclonal variation.

Biochemical Methods for the Characterization of Somaclonal Variation

Somaclonal variation may be evaluated by analysis of gene products rather than the genes themselves. Larkin and Scowcroft (1981) reviewed somaclonal variations in a range of biochemical constituents. The levels of specific metabolites or enzymes may be analysed to determine somaclonal variation. The most common application of such tests would be in the selection of useful variation in a specific metabolite. The most common analysis that aims to indirectly detect genetic

change is the assessment of isoenzymes. These are gene products but reflect genetic differences in most cases. The levels of secondary metabolites in plants have been studied in some detail because of the potential to use appropriate somaclonal variants as sources of specific metabolites of commercial value. This provides a very large range of compounds that can be used to characterize somaclonal variation.

Isozymes and RFLP were used to analyse variation in beet plants regenerated from tissue culture (Sabir *et al.*, 1992). The tests on more than 700 plants indicated that about 0.1% of alleles varied by both methods. In another study, isoenzyme and RFLP analysis gave consistent evidence for the lack of variation in embryogenic cultures of napier grass (*Pennisetum purpureum*), confirming the value of the propagation system (Shenoy and Vasil, 1992).

The number of possible isozyme systems is limited and usually no more than about 20 isozyme systems can be studied in any population. Some of the isozymes that have been used to monitor somaclonal variation include; acid phosphatase, alcohol dehydrogenase, aspartate aminotransferase, esterase, endopeptidase, glutamate dehydrogenase, hexokinase, malate dehydrogenase, malic enzyme, 6-phosphogluconate dehydrogenase, phosphohexose isomerase and shikimic dehydrogenase.

Orton (1980a) produced evidence for chromosomal substitutions in the production of double haploids of barley by using esterase isozymes.

Conclusions and Future Prospects

Molecular and biochemical characterization of somaclonal variation has great potential to identify somaclonal variation in different systems. Quantitative assessment of the relative rates of somaclonal variation may be obtained by molecular analysis and used for modifying culture conditions to either eliminate somaclonal

Table 3. Influence of plant hormones on the methylation of cytosine (LoSchiavo *et al.*, 1989)

Hormone	Concentration (mg/L)	Percentage methylation 5-methyl cytosine/total cytosine
2,4-D	0.5	16
	2	40
	5	45
NAA	2	19
	5	23
IAA	2	19
	5	21
BAP	2	16

2,4-D = 2,4 dichlorophenoxy acetic acid; NAA = naphthaleneacetic acid; IAA = indoleacetic acid; BAP = 6-benzylaminopurine.

variation or achieve an optimal level of variation. Specific high-frequency somaclonal variants may be identified using linked molecular markers. The genetic changes causing the somaclonal variation can ultimately be determined using the techniques of map-based cloning. The increasing knowledge of plant genome structure and improvements in technology for gene mapping and DNA sequencing will assist the detection of somaclonal variants. Complete sequences of mitochondrial and chloroplast genomes can now be compared to totally define somaclonal variation in these genomes. Detailed maps of plant nuclear genomes are being developed and databases of sequences of plant genes are growing rapidly. Molecular assessment of somaclonal variation will benefit from the availability of these data as a background against which somaclonal variation can be measured.

The ability to better characterize somaclonal variants using molecular techniques may allow more efficient exploitation of somaclonal variation as a source of genetic variation for crop improvement. Current molecular techniques permit the specific selection for any defined DNA sequence in highly efficient and simple screening protocols. An improved understanding of the structure of plant genomes and the assignment of known functions to an increasing proportion of plant genes will allow the general application of this technique to select desirable genotypes. The high level of synteny being revealed by the molecular characterization of plant genomes suggests that these techniques will find application even in currently less-well-studied plant species.

Biochemical and molecular methods allow characterization of the phenotypic and genotypic variation in somaclonal variants. Somaclonal variation may result from genetic or epigenetic changes that can be identified through various biochemical and molecular approaches. Genetic changes may occur in the nuclear, mitochondrial or chloroplast genomes, requiring the application of appropriate techniques. Isoenzyme and restriction fragment length polymorphism (RFLP) analyses were the first techniques to be widely used to characterize somaclonal variation. More recently, random amplified polymorphic DNA (RAPD) techniques have been applied to the analysis of somaclonal variation. New techniques such as amplified fragment length polymorphism (AFLP) analysis also have potential in the study of somaclonal variation. A range of techniques used for the molecular characterization of plant genomes have value in the investigation of somaclonal variation. Advances in mapping of plant genomes and the sequencing of plant genes are providing a continually growing database which allows somaclonal variation to be measured and defined at the molecular level.

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25. Recombinase-mediated Gene Integration in Plants

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Introduction

Genetic transformation of plants is potentially the most precise technology available for crop improvement. Compared to crossing in a trait or inducing mutations, introducing a single characterized gene, or even several genes in a biosynthetic pathway, would be a more direct way to alter a plant phenotype without incorporating unwanted changes. While important scientific and agricultural advances are being made through plant transformation, the existing procedures leave room for improvement. As with animal systems (Jaenisch *et al.*, 1981; Lacy *et al.*, 1983), the same transforming molecule does not always generate transgenic materials with the same phenotype (Herrera-Estrella *et al.*, 1984; Jones *et al.*, 1985). In some reports, a high degree of phenotypic variation is seen among independent transformants. In other cases, plants which initially expressed a transgene either did not express or showed a reduced level of transgene expression later in development or in subsequent generations (Brandle *et al.*, 1995; Meyer, 1995a,b).

Phenotype variation in transgenic plants has been attributed to a variety of causes: (i) mutations in the transforming DNA generated during transformation, (ii) variation in transgene copy number, (iii) intergenic interactions such as homology-dependent gene silencing, (iv) somaclonal variation, and (v) chromosome position effect.

During the transformation process, it is possible that transgene constructs could acquire mutations such as deletions or other rearrangements of the DNA. These mutations, which could significantly influence expression, have in some studies been attributed as the major source of variable transgene expression (Battacharyya *et al.*, 1994).

Transformed plants often contain variable numbers of transgenes at the same locus or in different loci. While in some cases transgene expression increases with more copies, a directly proportional relationship is rarely seen. In many cases, reduced gene expression has been associated with multiple copies of a transgene, to the extent that there is sometimes complete silencing of the transgene. Expression of endogenous genes homologous to the transgene may likewise be negatively affected. The structure of multiple insertion events may be important in determining the nature of the interactions between multiple transgenes. Single-copy insertions at multiple loci may be expressed quite differently from multiple copies at a single locus. The orientation (i.e. head-to-head, head-to-tail, tail-to-tail or mixed) of multiple copies at a single locus may also be important in determining gene expression. While the mechanisms of 'co-suppression' or

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'homology-dependent gene-silencing' are not fully understood, numerous workers have reported an apparent correlation between transgene copy number and incidence of reduced gene expression (Matzke and Matzke, 1995; Park *et al.*, 1996).

Somaclonal variation has been defined as the genetic variation displayed in tissue culture-regenerated plants and their progeny (Larkin and Scowcroft, 1981; Maretzki, 1987). Chromosome rearrangements, higher rates of mitotic crossing over, changes in repetitive DNA copy number and activation of transposable elements have been associated with somaclonal variation (Evans. 1989; Peschke and Phillips, 1992). While tissue culture-induced changes have been exploited by plant breeders as a source of genetic variability, somaclonal variation can be the bane of molecular biologists trying to make comparisons among different gene expression constructs, or attempting to engineer a specific trait.

Position effects could be defined as those factors associated with the particular chromosomal location of the transgene which influence its expression. Some chromosomal structures such as telomeres (Stavenhagen and Zakian, 1994) or heterochromatin (Howe *et al.*, 1995; Wallrath and Elgin, 1995) have been shown to affect the expression of nearby genes. In other cases where transgene expression has been dramatically different among independent transformants, specific chromosome structures influencing gene expression have not been identified. Since in most transformation systems the site of transgene integration is random, these influences on transgene expression may differ significantly among independent transformants.

Attempts to Control Gene Expression Variability

Efforts have been made to normalize transgene expression by incorporating two different reporter gene cassettes into the same transformation construct. If the chromosomal location of transgene integration were the main cause of variation, the expression of the two reporter genes relative to each other would be similar among different transformants. However, in several independent experiments, the expression from two reporter genes in a single construct was found to have widely different relative levels of expression among independent transformants (An, 1986; Dean *et al.*, 1988; Dunsmuir *et al.*, 1987; Peach and Velten, 1991). Other expression vectors have utilized a reference gene homologous to the test construct; based on the hypothesis that if some type of sequence-specific interaction were responsible for inconsistent expression ratios between different reference and test genes in one construct, then using a reference gene homologous to the test gene might produce reduced variation. With this strategy, Bruce and Gurley (1987) reported less than 10% variability in relative transcript levels between replicate experiments for the same promoter construct. However, the analysis was performed on pooled RNA from 200 to 300 transgenic crown gall tumours, and it is difficult to assess whether the low variability in gene expression is due to the design of this reference vector or the large number of individuals in each sample pool, or both.

The use of nuclear matrix attachment regions (MARs, or scaffold attachment regions) is another approach to reducing transgene expression variability. MARs are A/T-rich DNA elements typically found in non-transcribed regions of genes which are strongly bound by nuclear matrix protein preparations (Avramova *et al.*, 1995; Spiker and William, 1996). Chromosomal DNA is bound to the nuclear matrix at MARs with the intervening DNA forming loops between the attachment regions. MARs are thought to influence gene expression by isolating DNA within the loop from neighbouring domains, leaving within-loop DNA accessible to the transcriptional machinery (Van der Geest *et al.*, 1994). Functional characteristics of MARs may be conserved between fungi, animals and plants.

Fungal (Allen *et al.*, 1993), animal (Mlynárová *et al.*, 1994) and plant MARs have been tested in plant transformation experiments. These were reported in many experiments to have reduced transgene expression variability between independent transformants. However, their use was sometimes accompanied by anomalously higher or lower levels of transgene expression. For example, tobacco plants transformed with a construct containing both upstream and downstream MARs from the French bean β-phaseolin gene showed less variability of transgene expression, but higher expression levels than plants transformed with constructs lacking the MARs (Van der Geest *et al.*, 1994). In contrast, tobacco transformed with MARs from a soybean lectin gene showed reduced variability of transgene expression, but a lower expression level than plants transformed with the MARs-deficient construct (Breyne *et al.*, 1992). Some experiments produced mixed results. For example, tobacco plants were found to have increased levels of expression from a transgene using soybean heat-shock gene MARs, but variation among transformants was not significantly reduced (Schöffl *et al.*, 1993).

Homologous recombination can also be considered as a way to reduce transgene expression variability by targeting DNA into defined chromosome locations. In theory, it offers the potential for modifying the genome in a precise fashion. Homologous recombination between introduced DNA and the genome has been successfully employed in bacterial, fungal and mammalian systems. However, until recently, efforts to use homologous recombination in plants were met with such low efficiencies as to be impractical for most purposes (Baur *et al.*, 1991; De Groot *et al.*, 1992; Lee *et al.*, 1990; Offringa *et al.*, 1992). Recently, integration of a marker gene into the *Arabidopsis TGA3* gene was achieved through homologous recombination (Miao and Lam, 1995). Two homologous integration events were recovered from about 2600 transformed calli. While still lower than the efficiencies reported in fungal or mammalian systems, the successful recovery of these calli offers encouragement for homologous recombination in plant transformation. Unfortunately, efforts to regenerate plants from the transformed calli were unsuccessful. Thus, the effects of homozygosity of the modified *TGA3* allele could not be observed. It should be noted that homologous recombination may not necessarily lead to precise integration events. In one report, homologous recombination was successfully used for marker rescue of a defective kanamycin-resistance gene in transgenic tobacco. However in four of the five homologous transformants recovered, rearrangements occurred at the target locus (Risseeuw *et al.*, 1995).

Recombinase-mediated Transformation

Site-specific recombination systems can be used to control transgene insertion events. While the intent of this chapter is to review the use of site-specific recombination systems in plant transformation, relevant research has also been done in other systems. To more thoroughly present the current state of knowledge in this area, results from non-plant systems will also be discussed.

Several site-specific recombination systems from bacteriophage or yeast function in plant cells: Cre-*lox* from bacteriophage P1 (Dale and Ow, 1990; Odell *et al.*, 1990), FLP-*FRT* from *Saccharomyces cerevisiae* (Lyznik *et al.*, 1993), R-*RS* from *Zygosaccharomyces rouxii* (Onouchi *et al.*, 1991), and Gin-*gix* from bacteriophage Mu (Maeser and Kahmann, 1991). Each of these systems requires a specific asymmetric DNA recognition site (*lox, FRT, RS* or *gix*) and a single recombinase enzyme (Cre, FLP, R or Gin) which catalyses recombination between two sites. The wild-type Gin-*gix* system requires an additional protein, FIS; however a mutant Gin is able independently to catalyse recombination events between *gix* sites (Klippel *et al.*, 1988). Structurally these site-specific recombination systems are similar in that the minimum recombination sites are 34 bp or less, and consist of inverted repeats flanking an asymmetric spacer sequence (Ow and Medberry, 1995 and references therein). For Cre-*lox* (Hoess and Abremski, 1990; Mack *et al.*, 1992), FLP-*FRT* (Bruckner and Cox, 1986) and Gin-*gix* (Maeser and Kahmann, 1991) there is evidence that the inverted repeats act as binding elements for the recombinase enzymes, and that each inverted repeat binds one recombinase monomer. In this chapter we will refer to the inverted repeats or recombinase binding-elements as left and right 'elements'.

In these systems, recombination between two sites in opposite orientations on the same DNA molecule results in the inversion of the intervening DNA (Fig. 1A). Two sites on separate DNA molecules can recombine to exchange flanking DNA (Fig. 1B), or if at least one of the molecules is circular, recombination results in the integration of the two molecules. As these reactions are freely reversible, the product of such an integration event contains two recombination sites in the same orientation which can recombine again to excise the intervening DNA (Fig. 1C). Excision resulting from recombination between two sites on the same DNA molecule is kinetically favoured over integration which requires recombination between different molecules.

Intermolecular Recombination

In higher eukaryotic cells, site-specific recombination between different DNA molecules was first reported in tobacco protoplasts (Dale and Ow, 1990). One plasmid containing a cauliflower mosaic virus 35S RNA promoter (*35S*) followed by a single *lox* site was introduced along with a plasmid bearing a promoterless luciferase (*luc*) gene with a *lox* site at its 5′ end. In the presence of a *cre* expression construct, luciferase activity was detected in extracts made

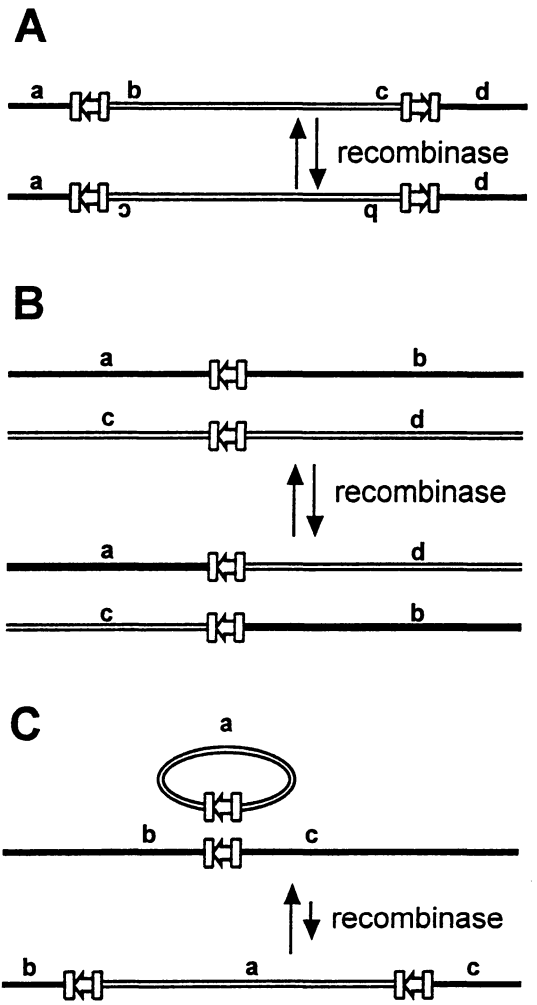

Figure 1. Site-specific recombination between recombination sites in different relative orientations. Recombination sites are represented by open arrows flanked by rectangles, where the open arrow represents the asymmetric spacer, and rectangles represent the right and left inverted repeats, or binding elements. Solid arrows represent relative rates of the reversible recombination reactions. Lower-case letters represent genetic markers. **A**: Recombination between sites in opposite orientations on the same molecule resulting in inversion of the intervening DNA. **B**: Recombination between sites on separate linear molecules, resulting in the exchange of flanking markers. **C**: Recombination between sites on a circular and a linear molecule, resulting in integration of the circular molecule. The reverse reaction, excision, is kinetically favoured over integration

from the protoplasts, indicating that intermolecular recombination joined *35S* to *luc*. Similar episomal inter-molecular recombination experiments were reported in maize and rice protoplasts using the FLP-*FRT* system (Lyznik *et al.*, 1993).

Insertional Recombination at Chromosomal Sites

Sauer and Henderson (1990) first reported using a site-specific recombination system to integrate DNA into eukaryotic chromosomal sites. 'Target constructs' containing a *lox* site were placed into the yeast or mouse genome. Transgenic cells then underwent a second round of transformation, using Cre recombinase to mediate the integration of an exogenous plasmid. While some transformed colonies contained multiple insertions at the chromosomal target site, and/or additional insertion events elsewhere in the genome, the predominant type of recombination event recovered was a single copy of the insertion plasmid integrated at the chromosomal *lox* target, without apparent rearrangements and without additional insertions elsewhere in the genome. In a similar strategy, site-specific integration in a monkey kidney cell line was similarly achieved using the FLP-*FRT* system (O'Gorman *et al.*, 1991).

In plants, Albert *et al.* (1995) used the Cre-*lox* system to direct the integration of plasmid DNA into chromosomal *lox* sites in tobacco. Target constructs containing *35S* immediately 5′ of *lox* were placed into tobacco chromosomes through *Agrobacterium*-mediated transformation. Protoplasts from these transgenic plants were then transformed with a promoterless selectable marker gene immediately 3′ of a *lox* sequence. Whole plants were then regenerated from transformed calli. This regeneration of whole organisms derived from site-specific integration of a transgene opens the possibility of monitoring transgene expression through organismal development and meiotic transmission.

Stabilization of Recombination Events

The reversibility of the site-specific recombination reactions makes the recovery of insertion events inherently difficult. Many insertion events are likely to be lost in the absence of a mechanism to stabilize them. To overcome this inherent instability, two general approaches have been used. The first relies on abolishing recombinase activity after DNA integration. The second relies on generating new recombination product sites that are inefficiently recognized by the recombinase.

Control of Recombinase Activity

Recombinase activity is required to catalyse DNA insertion, but immediately thereafter, it is a threat to the stability of the insertion. An inducible promoter driving expression of the recombinase gene was used to generate site-specific insertions in *S. cerevisiae* (Sauer and Henderson, 1990). Cre recombinase was produced from a plasmid in the target cells using an inducible promoter. After site-specific integration, the yeast cells were moved to growth medium which represses *cre* expression. Similarly, insertional recombination was achieved at *FRT* sites in the chromosome of *E. coli* when FLP recombinase production was controlled by a temperature-sensitive promoter (Huang *et al.*, 1991).

Transient expression from a non-replicating plasmid has also been used on numerous occasions to supply Cre recombinase for site-specific integration in a mouse cell line (Sauer and Henderson, 1990), a monkey cell line (O'Gorman *et al.*, 1991) and in tobacco protoplasts (Albert *et al.*, 1995). Cells that harboured chromosomal recombination sites were co-transformed with an insertion plasmid along with a recombinase expression plasmid which could not replicate. Provided that the recombinase plasmid did not integrate, production of recombinase would cease, and cells with an integrated DNA molecule could be recovered as stable transformants.

Transient provision of recombinase can also be achieved through protein transformation (Baubonis and Sauer, 1993). Cultured human cells containing a chromosomal *lox* site were co-transformed with a *lox*-containing plasmid and purified Cre enzyme. The use of recombinase protein eliminated the possibility of sustained recombinase activity. However, the efficiency of transformation was not higher than that using recombinase expression constructs.

Transient provision of recombinase can also be achieved through genetic disruption of a recombinase-producing gene. Albert *et al.* (1995) used a chromosomal target construct in which insertional recombination would split the promoter from the *cre* coding region. Plants were stably transformed with constructs containing *35S* fused to *cre* with an intervening *lox* site. Protoplasts from these plants were then retransformed with a promoterless *lox*-hygromycin phosphotransferase (*hpt*) plasmid. Site-specific recombination at the chromosomal *lox* site resulted in a *35S–lox–hpt* fusion, conferring hygromycin resistance, and displacement of the *cre* gene from its promoter, which terminates *cre* transcription (Fig. 2A).

Mutant Recombination Sites

Recombination sites can be designed to favour insertional but resist excisional recombination. This approach was tested with the FLP-*FRT* system (Senecoff *et al.*, 1988). The *FRT* recombination site consists of two 13 bp (imperfect) inverted repeats flanking an 8 bp asymmetric spacer. FLP recombinase is thought to act as a dimer, with one FLP monomer at each *FRT* binding element. These binding elements consist of the innermost 11 bp of the inverted repeats, and the first (outermost) base of the spacer region. A large collection of single-base mutations in the FLP binding element were synthesized and tested for recombination efficiency *in vitro*. Recombination between two single-mutation *FRT* sites, where the mutations are on opposite elements, would result in one product site with mutations in both elements and the other product site fully wild-type (Fig. 2B). In one particular base change, the recombination between two single-mutation *FRT* sites, where the mutations are on opposite elements, occurred less efficiently than between wild-type sites; but much more efficiently than the recombination between product sites generated by this recombination reaction. This suggested that the product site with mutations in both binding elements is not efficiently recognized by the recombinase. Hence, when these *FRT* sites with single mutations are used for insertional recombination, the integrated molecule would be less likely to

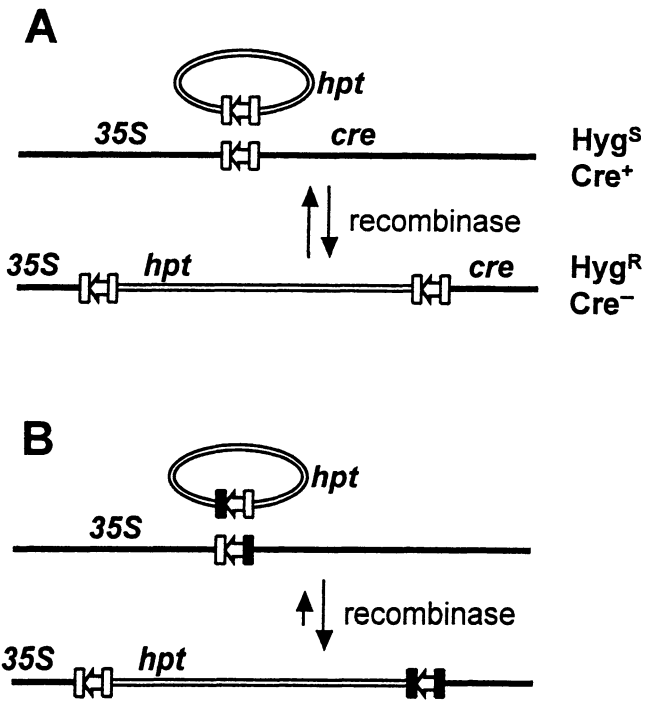

Figure 2. Methods of stabilizing integration events. **A**: Termination of recombinase gene expression by promoter displacement. **B**: Mutant recombination sites designed to shift the integration–excision equilibrium. Empty rectangles represent wild-type recombination site binding elements, black rectangles represent mutant binding elements (Albert *et al.*, 1995)

excise. When compared against wild-type sites for DNA integration into an *E. coli* chromosomal target, however, the mutant pair of *FRT* sites produced 100-fold fewer integration events than did the wild-type sites. It is possible that low levels of FLP activity present in the host cells could not catalyse efficient recombination between the pair of mutant *FRT* sites (Huang *et al.*, 1991).

Like the FLP-*FRT* system, Cre recombinase binding to the inverted repeats of a *lox* site appears to have a strong cooperative nature (Hoess and Abremski, 1990; Mack *et al.*, 1992), suggesting that the same principle of introducing mutations into the binding elements could be applied. Mutant recombination sites were successfully used to mediate insertional recombination at *lox* sites placed in the tobacco genome (Fig. 2B). When Cre recombinase was expressed from a *cre* gene in the genomic target locus where insertional recombination displaced the *cre* gene from its promoter, no recombination events were recovered using wild-type *lox* sites, but some were recovered with the use of mutant sites. When Cre was supplied by transient expression from a co-transformed plasmid, integration events were recovered with approximately equal frequency using either mutant or wild-type *lox* sites (Albert *et al.*, 1995). Possible 'leaky' expression of the displaced *cre* gene, or slow turnover of a large pool of Cre recombinase resulting

from prior *cre* expression from the target locus, may have led to greater instability using wild-type sites. *In-vitro* comparisons of wild-type and double-mutant *lox* sites showed the greatest difference in 'forward' (insertion) versus 'reverse' (excision) recombination at low concentrations of Cre (Albert *et al.*, 1995).

Efficiency of Insertional Recombination

The efficiency at which insertional recombination occurs is important in how widely applicable this technology may be. Intermolecular recombination between extrachromosomal plasmids introduced into tobacco protoplasts by electroporation occurred at levels approaching those of intramolecular recombination events, when amounts of the *lox* site-containing substrate plasmids and of the *cre* expression plasmid were optimized (Dale and Ow, 1990). As electroporation can result in the introduction of numerous plasmids into each cell, the probability of *lox* sites on separate plasmids interacting is significantly higher than an interaction between a plasmid *lox* site and a single chromosomal *lox* site.

In yeast, Cre-*lox*-mediated insertional recombination occurs more frequently than random integration, but five- to ten-fold less than homologous recombination, which is an efficient process in *S. cerevisiae*. In two experiments, seven and 23 Cre-*lox* mediated insertion events were recovered from 10 million cells. When this plasmid was linearized to enhance homologous recombination in yeast, 53 and 92 transformants were recovered (Sauer and Henderson, 1990).

When cultured mouse cells with chromosomal *lox* sites were transformed with a plasmid containing a *lox* site, the number of transformed colonies per million cells averaged 116 (range 32–253, $n = 3$). In conventional (random integration) transformation, the same cells yielded an average of 299 (range 191–423, $n = 4$) (Sauer and Henderson, 1990). These experiments suggest that Cre-mediated insertion at a chromosomal *lox* site occurs at rates ranging from equally efficient to ten-fold less efficient than random integration. In assessing the efficiency of site-specific integration, it may be of significance to note that the mouse cell line used had multiple copies of chromosomal *lox* target sites rather than a single copy (Sauer and Henderson, 1990).

Cultured monkey cells containing chromosomal *FRT* sites were transformed with a *FRT* site-containing plasmid (O'Gorman *et al.*, 1991). In two experiments, 46 of 76 and 58 of 82 transformed colonies displayed phenotypes consistent with integration of the plasmid at the chromosomal *FRT* site. Transformation with plasmids lacking the *FRT* site yielded 23 and 32 transgenic colonies. This suggests that the FLP-*FRT*-mediated integration may occur at rates equal to or greater than random integration. While it is difficult to compare recombination efficiencies in experiments which differ in many respects (cell line, method of scoring recombination, etc.), the authors suggested that FLP-*FRT* may be more efficient than Cre-*lox* in mediating recombination in mammalian cells.

The efficiency of Cre recombinase-mediated integration of DNA at a chromosomal *lox* site was compared to random-site integration in tobacco, using

polyethylene glycol-mediated transformation of protoplasts (Albert *et al.*, 1995). While transformation efficiencies varied widely among experiments, highest efficiencies of Cre-*lox*-mediated transformation were about equal to efficiencies for random integration, but most often were about ten-fold lower. Transformation efficiencies for wild-type *lox* sites versus mutant *lox* sites designed to stabilize integration events were compared. When *cre* was expressed from a chromosomal locus, no stable integration events were recovered at wild-type *lox* sites, but some were recovered from mutant *lox* sites. When *cre* was expressed transiently from a co-transformed plasmid, mutant and wild-type *lox* sites yielded comparable numbers of stable transformants.

DNA Rearrangements Accompanying Site-specific Recombination

Site-specific recombination systems function conservatively within their natural systems, i.e., recombination events occur without the loss, addition, or other rearrangement of the participating DNA. If this property were retained during transformation, this in itself would be an important advance. Existing transformation methods often incur significant rearrangements in the transforming DNA, which may have major effects on the expression of transgenes. In yeast, analysis of Cre-*lox*-mediated insertion at chromosomal *lox* sites indicated that four of 15 events had tandem insertions, and some of these events had more than two tandem copies (Sauer and Henderson, 1990).

In mouse cells, eight insertions at chromosomal *lox* sites were analysed by Southern blots; one was a tandem insertion but the rest were single copy (Sauer and Henderson, 1990). Analysis of these events was complicated by the presence of several chromosomal *lox* target loci in the host cell genome, and it appears that at least one of these eight events may have undergone rearrangements of the recombined locus. Southern analysis of 56 Cre-*lox*-mediated insertions at chromosomal *lox* sites in cultured Chinese hamster ovary cells detected only one rearrangement in the post-recombination locus (Fukushige and Sauer, 1992). The analysis included examining the upstream junction fragment that produced the functional selectable marker gene transcription unit, but failed to examine the downstream junction. It is possible that higher rates of rearrangements would be detected at the downstream junction, since alteration at this region would have a negligible effect on the selected phenotype.

PCR analysis of both junction fragments formed by Cre-*lox*-mediated integration in tobacco detected no rearrangements at the upstream junction that formed the selectable marker gene. However, at the downstream 'non-selected' junction, only 44 out of 60 analysed integration events showed a precise integration pattern (Albert *et al.*, 1995). Southern analysis of several of these imprecise integrant plants confirmed that rearrangements had occurred. Nevertheless, the overall fidelity of integrated transgenes remained high, with almost 75% showing no rearrangements detectable with PCR or Southern hybridization analysis. Rearrangements were not reported in the eight FLP- mediated insertions at a chro-

mosomal target *FRT* site in cultured human embryonic kidney cells (O'Gorman *et al.*, 1991).

Random Integration Events Accompanying Site-specific Events

As site-specific integration events generally appear not to occur at frequencies higher than random insertion events, it would seem that random integrations might frequently accompany site-specific integrations. However, this does not appear to be the case. In yeast, some tandem insertions were seen at a chromosomal *lox* site, but insertions of the integration plasmid elsewhere in the genome were not detected following Cre-*lox*-mediated integration (Sauer and Henderson, 1990). In eight Cre-*lox*-mediated insertion events in cultured mouse cells that were analysed, Southern blotting revealed only one instance of a random insertion event accompanying site-specific integration (Sauer and Henderson, 1990). Other experiments in Chinese hamster cells found only two random insertions accompanying 56 Cre-*lox*-mediated site-specific integration events analysed by Southern hybridization (Fukushige and Sauer, 1992). Cre-*lox*-mediated insertional recombination in tobacco resulted in four random integrations accompanying site-specific integrations from 14 plants analysed by Southern blotting (Albert *et al.*, 1995). Southern blotting of eight FLP-*FRT*-mediated insertion events in human cells failed to reveal illegitimate recombination events accompanying the site-specific insertions (O'Gorman *et al.*, 1991). The collective experience thus far indicates that transgenes chromosomally integrated by site-specific recombination are in the great majority of cases not accompanied by random insertions.

'Position Effects' in Site-specific Recombination

A primary motivation for the development of site-specific recombination systems as transformation tools has been to reduce position effects on gene expression. It has been proposed that there may also be 'position effects' on the efficiency of site-specific recombination: recombination at one site within the genome may occur more readily than at other sites in the genome. Different Chinese hamster cell lines with single copy chromosomal *lox* targets were found to have dissimilar efficiencies for generating site-specific integration events (Fukushige and Sauer, 1992). Similar experiments in independent human cell lines containing chromosomal *lox* targets indicated that *lox* sites at different chromosomal locations may undergo recombination at significantly different rates (Baubonis and Sauer, 1993). Copy number of the chromosomal *lox* target sites in these lines varied from 2 to approximately 60, mostly as tandem arrays. The number of transformants recovered varied more than 60-fold among different cell lines, but the number of transformants was not correlated to the number of *lox* target sites. In fact, the highest number of transformants was recovered from a cell line with one of the lowest number of *lox* sites (about four). The number of transformants per *lox* target site

differed by more than 15-fold. Unfortunately, the presence of multiple *lox* target sites in the cell lines makes the data more difficult to evaluate. It seems possible that tandem arrays of *lox* target sites, which accounted for most of the multiple copies, would be resolved to single copies by excisional recombination in the presence of Cre recombinase. As molecular analysis of the integrations was not reported, the final number of *lox* target sites after an integration event is not known.

Reproducible Gene Expression at Specific Chromosomal Sites

The bottom-line question about these transformation systems is whether or not they do in fact reduce the variation in transgene expression among independent transformants. So far there is only one report addressing this issue, and the data are encouraging (Fukushige and Sauer, 1992). Two independent hamster cell lines were generated, each carrying a single copy chromosomal *lox* target. The cells were transformed with insertion plasmids carrying a β-galactosidase gene controlled by a human β-actin promoter. Colonies derived from independent insertion events which lacked multiple insertions or gross rearrangements, were quantitatively assayed for β-galactosidase activity. With few exceptions, single copy insertions in the same cell line showed quite similar levels of β-galactosidase activity. The greatest variation among independent transformants was less than a five-fold difference, whereas differences up to 100-fold and more have been reported with conventional transformation systems in mammalian cell cultures (Phi-Van *et al.*, 1990). Still higher levels of transgene variation have been reported in whole animals (Furth *et al.*, 1991) and whole plants (An, 1986).

With one insertion plasmid, the average β-galactosidase activity of transformants from one cell line differed from that of the other cell line at the 99% confidence level. A different insertion plasmid yielded activity levels which were not significantly different between transformants of the two independent cell lines. In this experiment, expression differences among transformants obtained from one cell line were greater than the difference between the averages of the two groups. While these experiments show a significant improvement in reproducibility of transgene expression with the use of a site-specific recombination system, the data also indicate that some significant part of gene expression variation among independent transformants may have causes other than the site of integration. What effect this may have on variability of gene expression in differentiated organisms, as opposed to cultured cells, remains to be seen.

Future Prospects

In recent years, homologous recombination has been used to place recombination sites in mammalian chromosomes (Fassler *et al.*, 1995; Zou *et al.*, 1994). While

these experiments used site-specific recombination to generate deletions in the genes targeted by homologous recombination, they demonstrate the potential for combining homologous and site-specific recombination into one transformation system. By using homologous recombination, a target recombination site could be placed in or near a specific endogenous gene. Once this target site has been established, the fidelity of site-specific recombination could be used to direct any number of modifications to the same chromosome position.

Serial targeting of multiple transgenes to the same locus may be feasible, perhaps by using a combination of different site-specific recombination systems. For example, an insertion plasmid for Cre-*lox*-mediated transformation could carry a *FRT* site, in addition to the gene of interest and a *lox* site. The *FRT* site could then act as a target site for a subsequent round of FLP-*FRT*-mediated transformation, which might install another gene of interest and an *RS* site for still another round of transformation. Engineered plants in which new traits are added to the same locus would greatly facilitate the introgression of these genes into elite cultivars that are difficult to transform.

Large DNA libraries, such as yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) libraries, are powerful tools for mapping and isolating novel genes. Methods which facilitate transformation with these large clones will help realize their full potential. Cloning vectors which incorporate site-specific recombination sites along with an appropriate selection scheme for detecting site-specific integration may provide a way by which large DNA fragments can be introduced into plant cells with a minimum of rearrangements. This would make it possible to screen such libraries by complementing mutations. Quantitative trait loci and other difficult-to-identify genes could be introduced into plants without ever having been identified, so long as they could be mapped to a particular YAC or BAC clone.

Conclusion

Genetic transformation of plants is a powerful technology in basic plant research and applied agriculture. Inconsistent expression of transgenes, both among individual transformants, and during the development and differentiation of individual plants, is a drawback of conventional present-day plant transformation methods. Much of this inconsistency has been attributed to chromosome position effects. However, other factors, including transgene copy number and fidelity, epigenetic effects and somaclonal variation, may also result in transgene expression variation. Site-specific recombination has been demonstrated as a useful tool for controlling not only the site of chromosomal integration, but also transgene copy number and fidelity. It is not yet clear how much of the total variation in transgene expression can be reduced by the use of these systems, but initial experiments with cultured animal cells have produced encouraging results. Current research using this new tool is under way to address this question in whole plants.

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26. T-DNA Insertion Mutagenesis and the Untagged Mutants

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Introduction

Insertion mutagenesis is defined as the insertion of DNA of known sequence into a gene. In plants there are two types of insertion elements that have been used for this purpose: transposons and T-DNA. Numerous genes have been isolated from species such as *Zea mays* and *Antirrhinum majus* that harbour endogenous transposable elements. In species such as *Arabidopsis thaliana*, heterologous transposons have been used to mutagenize genes (Bhatt *et al.*, 1996). Transposon mutagenesis will be described elsewhere in this volume (Chapter 19).

In this chapter we will describe *Agrobacterium*-mediated transformation, *Arabidopsis* as a plant model system for gene tagging, T-DNA insertion mutagenesis, various transformation protocols for *Arabidopsis*, the library of insertion mutants that has been generated in *Arabidopsis*, and a PCR-based reverse genetics procedure that is being applied to this library. These will serve as background for a description of the putative somaclonal variants and untagged mutants that are generated by *Agrobacterium*-mediated transformation procedures. We will also discuss mechanisms by which untagged mutants are generated. Much of this discussion will be restricted to *Arabidopsis* as it is in this species in which the vast majority of the research has been conducted.

Agrobacterium-mediated Transformation

Agrobacterium tumefaciens is a plant pathogenic soil bacterium responsible for crown gall disease (Hooykaas and Beijersbergen, 1994). The host range for *A. tumefaciens* was for years believed to be restricted to the dicotyledonous species (DeCleene and Deley, 1976). However, recent reports make it clear that many, if not all, monocotyledonous plants are also susceptible to infection (Mooney *et al.*, 1991; Hiei *et al.*, 1994; Ishida *et al.*, 1996). Pathogenesis and virulence require the presence of a large tumour-inducing (Ti) plasmid in *Agrobacterium.* Located on the Ti plasmid is a region of DNA delimited by 25 bp imperfect direct repeats (T-DNA) that will be transferred to the plant cell. Genes located in the T-DNA are not required for T-DNA transfer and integration but rather are important for the synthesis of amino acid and sugar derivatives, termed opines. Also in the T-DNA are genes responsible for the synthesis or modification of auxins and cytokinins. Synthesis of these phytohormones leads to neoplastic

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growth in the plant resulting in crown gall disease (for a review of this subject see Hamill, 1993). These phytohormone and opine synthetic genes can be removed from the T-DNA and replaced with foreign genes that are useful for selection or scoring of the transformation event and/or plant improvement.

Facile *Agrobacterium*-mediated transformation systems have been described for a wide variety of species including *Nicotiana* (Matsumoto *et al.*, 1990); *Lycopersicon* (Chyi *et al.*, 1986); *Arabidopsis* (Feldmann and Marks, 1987; Valvekens *et al.*, 1988; Bechtold *et al.*, 1993); *Petunia* (Deroles and Gardner, 1988); *Zea* (Ishida *et al.*, 1996); *Oryza* (Hiei *et al.*, 1994) and many other species (for protocols see Potrykus and Spangenberg, 1995).

Arabidopsis as a Plant Model System for Gene Tagging

Arabidopsis is a nearly ideal organism for gene tagging because of its numerous biological and genetic attributes. Chief among these are its small size (25–35 cm at maturity), rapid life cycle (5–8 weeks from seed to seed), amenability to a wide variety of growth regimes and transformation procedures, small seed size (important for mutant screening), genetics (diploid and self-fertilizing), and small genome size (estimated to be 120 Mbp). Additional advantages include: various genetic mapping systems, large numbers of researchers focusing on this organism, and availability of biological resources through federally funded resource centres (for a review of *Arabidopsis* see Redei, 1975, 1992).

T-DNA Insertion Mutagenesis in *Arabidopsis*

Only in *Arabidopsis* have any genes been cloned utilizing T-DNA as a gene knockout. In fact, this approach has been so successful in *Arabidopsis* that genes have been cloned from T-DNA tagged mutants generated by a number of diverse transformation protocols. These include tissue culture as well as whole plant procedures. The various transformation systems are described below.

There are probably a number of reasons that T-DNA tagged mutants have not been isolated from other plant species. For some species, such as *Z. mays* and *O. sativa*, transformation has only recently been a reality and the number of total transformants is probably too low for a full appraisal. For other species, such as *Nicotiana tabacum*, the polyploid nature of the genome would make it difficult to find recessive mutants. Hayashi *et al.* (1992) circumvented this problem by screening for dominant mutants with a T-DNA that contained multiple transcriptional enhancers located near the right border. In this way they were able to identify a gene which when overexpressed permitted growth of tissue *in vitro* in the absence of exogenously supplied auxin. In many agronomic species where thousands of transformants have been examined, such as *Lycopersicon* spp. and *Petunia* spp., gene redundancy is a more significant problem than in *Arabidopsis*. Finally, variants isolated from transformation experiments were often discarded as

probable somaclonal mutants instead of being tested for cosegregation of the mutation and the selectable marker.

Tissue Culture Procedures

Traditionally, to regenerate plants from *Arabidopsis*, large masses of calli were generated on a callus-inducing medium (CIM; high auxin : low cytokinin) and then subdivided and transferred to shoot-inducing medium (SIM; low auxin : high cytokinin; Gresshoff and Doy, 1972; Negrutiu *et al.*, 1978a,b). Regeneration occurred at a very low frequency and sterility was often observed in regenerated plants. This loss in morphogenic capacity was hypothesized to result from chromosomal instability in established calli. Negrutiu *et al.* (1978a), for example, showed that callus derived from leaves or stems was more prone to chromosomal instability than callus derived from seedlings. They were able to attribute increases in chromosomal instability to exogenous phytohormones in the media used for callus maintenance and regeneration (for a review of this subject see Feldmann, 1992b). To circumvent the problem of chromosomal instability (somaclonal variation; see Lee and Phillips (1988) for a review) associated with long-term exposure to exogenous hormones and to increase the regeneration frequency, Feldmann and Marks (1986) developed a rapid regeneration system for *Arabidopsis* based on regeneration protocols developed for *Convolvulus arvensis* by Christianson and Warnick (1985). A short preculture (5–7 days) on CIM before transfer to SIM facilitated the regeneration of fertile whole plants from up to 100% of the explants in as little as 5 weeks from stem and leaf explants (Feldmann and Marks, 1986; Feldmann, 1992b). However, when *Agrobacterium*-mediated transformation was added to this protocol, the exposure to CIM was increased with a concomitant increase in somaclonal variation (K.A. Feldmann and M.D. Marks, unpublished results).

Valvekens *et al.* (1988) followed up on the short preculture concept of Feldmann and Marks (1986) to develop a transformation system for *Arabidopsis* using root explants, and observed high rates of transformation and plant regeneration. Again, the transformation and selection process meant longer exposure to exogenous hormones before regenerants were large enough to be transplanted. As a result, preliminary data from progeny of 60 root- or leaf-derived transformants showed that none of the visible mutations were linked to the T-DNA (Valvekens *et al.*, 1988). In a follow-up report, progeny from 101 root-derived regenerants (non-transformed) were found to segregate for seven visible recessive mutations while screening progeny from 84 transformants resulted in an additional seven lines segregating for recessive mutations; none of these latter lines appeared to be linked to the marker in the T-DNA (Valvekens and Van Montagu, 1990). These limited data strongly suggested that the seven mutants in the transformed population were due to somaclonal variation rather than a mutagenic event induced by the transformation process. An especially interesting result from this study was that, for the lines segregating for visible mutants, the ratio of wild-type to mutant plants was far higher than the 3 : 1 expected for progeny from a plant heterozygous for a

recessive mutation, suggesting that most of the regenerants were chimaeras, consisting of wild-type and heterozygous tissue. It is intriguing to speculate that the *Agrobacterium* which had survived the selection process remained in the interstitial tissues and were somehow responsible for inducing untagged mutations during the growth of the regenerant. As we will describe later, these same types of transformants are generated with whole plant transformation systems. The Valvekens *et al.* (1988) protocol is sufficient for introducing genes into *Arabidopsis* but it is not likely to be extremely useful for insertion mutagenesis.

Lloyd *et al.* (1986) utilized a leaf disc *Agrobacterium*-mediated transformation procedure to introduce a hygromycin-resistance marker into *Arabidopsis.* The generation of 100 transformants and their subsequent phenotypic analyses resulted in numerous mutants. However, none of the mutations cosegregated with the hygromycin resistance marker in the T-DNA (R. Horsch, personal communication). This result was assumed to be due to somaclonal variation induced during long periods of exposure to exogenous hormones through the transformation and regeneration process.

Van Lijsebettens *et al.* (1991b), using a modified protocol of Lloyd *et al.* (1986), regenerated 73 non-transformed plants and found that only one segregated for a visible alteration in phenotype. They also screened 110 leaf-derived transformants and found that 5% (6/110) of the transformants displayed an alteration in phenotype. One of these mutants, a leaf morphology mutant (*pointed first leaves, pfl*), cosegregated with the T-DNA. They have subsequently cloned this gene and shown, via molecular complementation, that it was tagged (Van Lijsebettens *et al.*, 1991a,b, 1994). For the other mutants, the T-DNA and mutation, at the level examined, were unlinked. These 110 transformants contained about 150 inserts such that the ratio of visible tagged mutations to T-DNA insertions (T-DNA mutation induction frequency) was extremely low, between 0.5% and 1%.

Redei *et al.* (1988) also developed a tissue culture-based transformation system for leaf and root explants that has proven to be moderately useful for insertion mutagenesis. Utilizing transformants generated with this protocol, Koncz *et al.* (1989) demonstrated that 30% or more of the T-DNA inserts induced gene fusions in *Arabidopsis*; they observed a similar percentage in *Nicotiana* transformants. They concluded that, because of the differences in genome size and density and distribution of transcribed genes, T-DNAs preferentially insert into transcribed units. They speculate that 30% is likely an underrepresentation as few organs were examined and even then only at certain developmental stages. Herman *et al.* (1990) reported that 70–100% of transformed calli from *Nicotiana* had at least one of their T-DNA insertion borders in a transcriptionally active region. For tobacco, this seems to be convincing evidence that T-DNA must preferentially insert into transcribed regions. These data make it even more surprising that no T-DNA tagged genes have been cloned from any species other than *Arabidopsis.* Given the high percentage of inserts that are found in transcribed regions, it might be interesting to clone and sequence a number of plant flanking DNAs from one of these species to verify the percentage that have inserted into transcriptional units.

Cloned Genes from Tissue Culture-derived Transformants of Arabidopsis

Using the Redei *et al.* (1988) protocol, Koncz *et al.* (1992) generated about 3000 stem-, leaf-, and root-derived transformants of *Arabidopsis.* Screening of 1340 of these segregating families showed that progeny from 25% of the primary transformants segregated for a visible alteration in phenotype. They estimate that as few as 10% of these might be tagged. These mutants were placed in phenotypic classes that were comparable to those generated by Feldmann (1991; Table 1).

Six genes have already been cloned from various tissue culture-derived transformants. Koncz *et al.* (1990) cloned *CH-42* from a tagged yellow–green mutant; this gene encodes a chloroplast protein. More recently, a second gene, *CPD*, encoding a cytochrome P450 sterol hydroxylase, was cloned from a dwarf mutant from this population (Szekeres *et al.*, 1996). As described previously, only one gene, *PFL*, has been cloned from the Van Lijsebettens *et al.* (1991b) population; *PFL* encodes a S18 ribosomal protein (Van Lijsebettens *et al.*, 1994).

The other genes that have been cloned from tissue culture-derived transformants were identified in transformation experiments where T-DNAs were introduced for reasons other than directed insertion mutagenesis. Cloned genes include *GA4* (Chiang *et al.*, 1995), *AP2* (Jofuku *et al.*, 1994) and *DWF1* (referred to as *DIM1* by Takahashi *et al.*, 1995). For gene functions see Table 2.

Predictions for Insertion Mutagenesis in Tissue Culture-derived Transformants of Arabidopsis

From the Koncz *et al.* (1989, 1992) data we can speculate that in 100 transformants, containing 150 independent insertion sites, there should be a disruption in a minimum of 45 genes (30% of 150 inserts into transcribed regions). The

Table 1. Frequency of various classes of visible mutant phenotypes observed from the screen of 5000 transformed lines from seed infection/transformation Arizona population and 1340 transformed lines derived via tissue culture procedures*

Phenotype	Seed transformation (%)	Tissue culture (%)
Size variant	4.4	5.52
Pigment mutants	3.86	4.47
Morphological mutants	2.82	4.54
Embryo-defective	3.6	5.52
Physiological mutants: flowering time	0.48	0.44
Seedling–lethal	1.1	0.59
Reduced fertility	1.1	0.36
Other	2.12	3.56
Total	19.48	25.00

*Data are drawn from Forsthoefel *et al.* (1992) and Koncz *et al.* (1992).

Table 2. Genes cloned from Arabidopsis via T-DNA-mediated gene tagging (modified from Choe and Feldmann, 1998)

Phenotype – Gene symbol	Function	Citation
Pigment mutants		
*CH42**	Chloroplast protein	Koncz *et al.*, 1990
CLA1	Novel/transketolase	Mandel *et al.*, 1996
PAC1	Novel	Reiter *et al.*, 1994
Dramatic morphological mutants		
AG	Transcription factor	Yanofsky *et al.*, 1990
*AP2**	Nuclear protein; 68 aa repeat motif	Jofuku *et al.*, 1994
*CPD**	P450 sterol hydroxylase	Szekeres *et al.*, 1996
*DWF1**	Oxidase	B. Dilkes, unpublished results
DWF4	22α hydroxylase	Choe *et al.*, 1998
FEY	Nodulin/reductase	Callos *et al.*, 1994
GL1	Transcription factor – *myb*	Oppenheimer *et al.*, 1991
GL2	Homeo box	Rerie *et al.*, 1994
*PFL**	S18 ribosomal protein	Van Lijsebettens *et al.*, 1993
SAB	Novel	Aeschbacher *et al.*, 1995
TSL1	Serine-threonine kinase	Roe *et al.*, 1993
Embryonic mutants		
COP1	Transcriptional regulator	Deng *et al.*, 1992
COP9	Novel	Wei *et al.*, 1994a
EMB30	SEC7 homology	Shevell *et al.*, 1994
FUS6	Novel	Castle and Meinke, 1994
Phytohormone affected mutants		
AUX1	Amino acid permease	Bennett *et al.*, 1996
CTR1	Serine-threonine kinase	Kieber *et al.*, 1993
*GA4**	Hydroxylase	Chiang *et al.*, 1995
HLS	N-acetyltansferase	Lehman *et al.*, 1996
RCN	Protein phosphatase 2A	Garbes *et al.*, 1996
Biochemical mutants		
CER1	Histidine-rich motifs	B. Lemieux, personal communication
CER1-like	Histidine-rich motifs	Deng *et al.*, 1996
CER2	Novel	Negruk *et al.*, 1996
CER3	Novel	Hannoufa *et al.*, 1996
FAD2	Fatty acid desaturase	Okuley *et al.*, 1994
FAD3	Fatty acid desaturase	Yadav *et al.*, 1993
Environment mutants		
HY3	Phytochrome B	Reed *et al.*, 1993
HY4	Microbial DNA photolyase	Ahmad and Cashmore, 1993
LD	Bipartate nuclear localization signal	Lee *et al.*, 1994
CHL1	Nitrate transporter	Tsay *et al.*, 1994
Other		
ANT1	AP2-like	Klucher *et al.*, 1996

*Isolated from tissue culture-derived transformant.
†Referred to as *DIM1* in Takahashi *et al.* (1995); *dim1* was cloned from a tissue culture-derived transformant.

Herman *et al.* (1990) data indicate that as many as 70–100 genes (70–100% of the calli) may have been disrupted in 100 transformants. Further, a conservative estimate of the percentage of genes that when disrupted would produce a visible alteration in phenotype (obvious with the naked eye or under the microscope but without any type of selection) is 5%. We derived this estimate from the T-DNA population as follows: 20% of the transformants (13% of the inserts; Table 1) show a visible alteration in phenotype. Of these only 40% are due to a functional insert (as assessed by kanamycin resistance). This means that 5% of the inserts caused a visible alteration in phenotype; thus in 100 transformed lines (150 inserts), there should be on average seven or eight tagged visible mutants. This is higher than what has been observed by Van Lijsebettens *et al.* (1991b) or Koncz *et al.* (1992). Still, the population sizes are small and they may only have examined a small set of especially interesting mutants among their transformed populations.

What seemed clear from all of the tissue culture-based transformation procedures was that it was going to be possible to clone genes via T-DNA tagging but that it was going to be a very laborious process to identify rare tagged mutants. Certainly, the identification and cloning of any gene of interest is not practical with this approach.

In-Planta *Procedures*

The first non-tissue culture transformation protocol, seed infection/transformation, was developed by Feldmann and Marks (1987). It has been the most successful to date for gene isolation due to the large number of transformants that have been generated and made available to the research community (Feldmann, 1991; Forsthoefel *et al.*, 1992). The procedures used are detailed elsewhere (Feldmann, 1991, 1992a, 1995; Feldmann *et al.*, 1994) and will only be outlined here. Briefly, 3000 wild-type seeds (T_1) were incubated with *Agrobacterium* (containing a marker for kanamycin resistance in the T-DNA) and the infected plants grown to maturity in bulk. The resulting progeny (T_2) were collected and screened for kanamycin-resistant (Kan^R) seedlings. The rare (about 1/5,000) Kan^R seedlings were transferred singly to soil and grown to maturity. Progeny (T_3) were collected from single plants, numbered chronologically, and subsequently screened for visible alterations in phenotype.

The idea for seed transformation came from the research of Ledoux *et al.* (1985). They reported the uptake of exogenous bacterial and human DNA into imbibing seeds of *Arabidopsis* and its eventual integration into the plant genome. This result led Feldmann and Marks (1987) to speculate that a motile *Agrobacterium* might be able to swim through the micropyle and up to the shoot apical meristem of an imbibed seed where it would attach and transfer T-DNA to the thin-walled, rapidly expanding, epidermal cells; these events would not result in transformed seeds unless cell replacement (an L2 cell replaced by a transformed epidermal cell) occurred. It was also proposed that the *Agrobacterium*, at

some low frequency, would reach the L2 layer and transform cells that would give rise to sporogenous tissue. Fortuitously, this latter assumption was incorrect (explained below).

Two other whole plant transformation techniques have been designed for the introduction of new constructs as well as insertion mutagenesis (Bechtold *et al.*, 1993; Chang *et al.*, 1994, Katavic *et al.*, 1994). In the Chang *et al.* (1994) procedure, young inflorescences were cut off near the base of soil-grown wild-type plants and the wounded surfaces at the base were inoculated with *Agrobacterium.* After the secondary inflorescences arose they were again cut off near the base and *Agrobacterium* was reapplied. Selfed seeds were collected at maturity and tested on the appropriate selective medium. Both C58 and LBA4404 strains of *Agrobacterium* were used successfully for this procedure (Coomber and Feldmann, 1993; Katavic *et al.*, 1994). However, this technique is probably not going to be sufficiently useful for insertion mutagenesis. There are at least two reasons for this prediction. First, because it is possible to generate a large sector of transformed tissue in a single plant, due to the possibility of the incorporation of a transformed cell in a newly forming meristematic region, the seeds from each treated plant will have to be collected separately to prevent contamination of the putative insertion population with siblings. Secondly, this technique is very labour-intensive. Despite this note of caution for insertion mutagenesis, this *in-planta* technique is a very useful transformation system for *Arabidopsis.*

An *in-planta* procedure which will be very powerful for saturating the *Arabidopsis* genome with T-DNA inserts was published by Bechtold *et al.* (1993). Wild-type plants (Wassilewskija; flowering with 7–10 cm peduncle) are submerged in an *Agrobacterium* suspension and subjected to vacuum infiltration. The plants are allowed to recover in the greenhouse and after several weeks seeds are collected. The seeds containing T-DNA inserts are germinated on kanamycin, selected, and grown to maturity. The authors estimate that they have generated > 50 000 transformants utilizing this procedure and are in the process of characterizing the first 12 000. This population is very similar to the transformants generated by seed infection/transformation, i.e., there are approximately 1.5 inserts/transformant, the inserts are concatamers of T-DNAs and there is a significant percentage of untagged mutants (M. Caboche, personal communication). This technique may not only prove to have solved the problem for saturation mutagenesis but is also useful for routine transformation; it is a robust transformation system that works in many laboratories.

As all of these transformation protocols must rely on the same mechanism, and as this may be pertinent to the generation of untagged mutants, it will be detailed here.

Mechanism of Transformation

The mechanism of transformation must account for (1) the generation of single transformed seeds from an infected plant, (2) the uniqueness of each transformant (i.e., no siblings), (3) the fact that 100% of the primary transformants (T_2) are heterozygous for the selectable marker and (4) a window during the imbibition

process when transformation is possible. We hypothesize that *Agrobacterium* cells enter through the micropyle, swim to exposed surfaces of the embryo, and enter interstitial spaces. The bacteria are carried along and possibly even divide in the plant as it develops. In the flower, the *Agrobacterium* are induced to transfer the T-DNA to a competent cell. We predict that the pollen- or megaspore-mother cells, their meiotic products, the products of micro- or megagametogenesis or the zygote are the target cells. It is possible that the apical cell of the embryo could be transformed just after the first mitotic division of the zygote as the untransformed basal cell will give rise only to the suspensor and a minor part of the root. Other than different means to introduce agrobacterium, we hypothesize that the mechanisms for transformation are the same in all whole plant transformation systems.

While wounding *per se* does not occur in the flower, there are unusual events which do occur that may produce chemicals that act as stimulants for *Agrobacterium* attachment and T-DNA transfer. Fertilization is one such event. Another would be pollen tube growth through the transmitting tissue to the ovule. Another might be during the selection of the megaspore with the resulting loss of the other meiotic products. The precise timing of transformation is unknown, but what is clear is that it happens in the flower of the T_1.

Feldmann and Marks (1987) showed that there was a window during the imbibition process when the seeds needed to be inoculated. If the bacteria were added to the imbibing seeds too early no or few transformants resulted, the same result as when the bacteria were added too late. Our observations of the infection process indicated that *Agrobacterium* were not able to enter the micropyle before the seeds had imbibed for a minimum amount of time, but instead attached to the integument. As more and more bacteria attached to the integument and to each other they appeared to create a barrier for subsequent entry through the micropyle when it opened.

Characteristics of the Transformed Population

More than 14 000 transformants have been generated with the seed infection/transformation protocol. Plating progeny (T_3) from primary transformants (T_2) on kanamycin has demonstrated that 57%, 25%, 5% and 5% contain one, two, three or four or more linked or unlinked inserts, respectively, and 9% segregate for a deficiency of Kan^R seedlings (exceptional segregants), with an average of 1.5 inserts/transformant. In subsequent generations, Kan^R progeny from these latter transformants were found to segregate for one to three linked or unlinked inserts with an average of 1.5 inserts per transformant. These transformants will be discussed in greater detail later. Thus, this population of transformants collectively contains 21 000 inserts in the genome.

Similar percentages were observed by Katavic *et al.* (1994) utilizing another *in-planta* procedure for *Arabidopsis.* Of 88 transformants, 60%, 16% and 15% segregated for one, two, and three or more inserts, respectively. Nine per cent segregated in an exceptional manner. For 44 transformants of *Nicotiana*, Budar *et al.* (1986) showed that 57% and 34% segregated for one and two inserts, respectively, while 10% segregated in an exceptional manner.

Characteristics of the Inserts

Many inserts consist of concatamers of T-DNAs in direct and inverted repeats about each border with more than 50% also containing a rearranged T-DNA. Castle *et al.* (1993) have diagrammed the insertion patterns in 10 embryo-defective mutants. Their analyses indicated that seven of the 11 insertions (one transformed line contained two linked insertions) were concatameric, each containing from two to five T-DNAs or parts thereof. Six of these insertions contained rearranged T-DNA. Among these 11 inserts, there are nine left border/plant flanking junctions (41%) and seven right border/plant flanking junctions (32%). The other six (27%) are internal T-DNA/plant junctions. From the genes that have been cloned thus far, where it is known which T-DNA border flanks the plant DNA, 64% (n = 16) are left border–plant junctions. As it can be difficult to show that an insert causing a particular mutation has internal T-DNA sequences as plant junctions, mutants that contain anything other than left and right border–plant junctions have not yet been characterized; these too will eventually be published. From analyses of insertion patterns of large numbers of published and unpublished transformants, we estimate that the average insert contains all or parts of three or four T-DNA units.

Mutant Spectrum

Because primary transformants (T_2) are heterozygous for the insert (selectable marker and mutation), progeny (T_3) will segregate 3 : 1 (R : S) for the dominant selectable marker (for a single insert), but 1 : 3 (mutant : wild-type) for any recessive mutations. Screening of 14 000 T_3 families under non-selective conditions with two separate environmental regimes, on soil in the greenhouse and on agar-solidified medium in vertically oriented plates (axenically grown), resulted in more than 2500 lines with segregating visible phenotypes. These mutants were placed in seven somewhat arbitrary phenotypic classes (Table 1). The majority of these lines were segregating 3 wild-type : 1 mutant, but a significant number were segregating for a deficiency of mutants. This latter observation suggested that a novel mutation occurred after the T_2 inflorescence meristem formed, giving rise to various-sized sectors of mutated sporogenous tissue and thus a variable but deficient number of mutants (a lower percentage of mutants than would be predicted by Mendelian principles; data not shown). Similar results were reported by Valvekens *et al.* (1988).

The screening procedures have been detailed elsewhere (Feldmann, 1991, 1992a; Choe and Feldmann, 1996). For brevity, the general phenotype in each class will be described and examples given of cloned genes if applicable. The functions of these genes are listed in Table 2. In addition, all of the untagged mutants that are known in each class will be listed.

Size variants are lines where small, spindly plants are segregating in a background of wild-type plants; dwarfs (Feldmann *et al.*, 1989) were placed in the morphological class (Table 1). Size variants possess small leaves, a reduced

stature and reduced seed set. These lines are likely to be metabolic mutants where a deficiency of some gene product causes the plant to be less vigorous. The frequency of mutants in this class was somewhat variable in that minor environmental perturbations could have produced smaller plants resulting in false positives. No genes have been isolated from mutants in this general class and little characterization of this class has been conducted.

Pigment mutants ranged from albinos, to yellow–green lethals, to plants that were initially green but turned yellow prematurely. Some of the mutants in this class are also somewhat unreliable in that the phenotype was not expressed in both soil-grown plants and in axenically grown plants for all lines. In addition, it can be difficult to discern whether a severe phenotype is yellow–green or albino. Mutants in this class can result from defects in the photosynthetic machinery, chlorophyll or carotenoid biosynthesis, and other types of biochemical lesions that may cause the mutant to be yellow–green. Several genes have been cloned from this class including *COP1, COP9, FUS6, PAC1*, and *CLA1* (Table 2). In addition to these tagged mutants, Norris *et al.* (1995) have identified and characterized two carotenoid deficient mutants, *pds1* and *pds2*, that failed to cosegregate with the Kan^R marker.

Morphological/dramatic mutants are those that have an altered organ/cell phenotype. These include flower, root, trichome, cell elongation (dwarf; Feldmann *et al.*, 1989), root, cotyledon and leaf mutants among others. A number of genes have been cloned from this class including *AG, ANT1, DWF1* (as *DIM1* by Takahashi *et al.*, 1995), *DWF4, FEY, GL1, GL2, SAB* and *TSL* (Table 2). Six untagged mutants with dramatic phenotypes have been characterized including two alleles of each of *SAB1* and *ANT1*, and one allele each of *GL2* and *LFY-7* (Table 3). Holding *et al.* (1994) have also described three root mutants which do not cosegregate with the Kan^R marker in the T-DNA. Other dramatic mutants have also been described for which nothing is known about the cosegregation of the T-DNA and the mutation (e.g., Feldmann, 1991; Jenks *et al.*, 1996).

Table 3. Untagged mutant alleles isolated from T-DNA-generated population

Gene symbol	Allele	Basis of mutation	Reference
ANT	*ant–1*	22bp deletion at nt 803	Klucher *et al.*, 1996
ANT	*ant–2*	G to A transition in exon 6	
CER2	*BRL7*	2 bp deletion and 4 bp addition in exon 2	Negruk *et al.*, 1996
CER2	*BRL17*	17 bp deletion in exon 2	
DET1	*det1–5*	9 bp in-frame deletion	Pepper *et al.*, 1994
GL2	*gl2–3063*	50 bp deletion spanning the junction-between exon 2 and intron 2	S.S. Sattler and M.D Marks; unpublished results
LFY	*lfy–7*	single bp change in exon 2 creating a stop	Weigel *et al.*, 1992
SAB1	*sable–2*	deletion of A at position 326 in exon 1	Aeschabacher *et al.*, 1995
SAB1	*sabl–3*	1.2 kb deletion of exons 6–8	

Embryo-defectives include mutants in which the embryo fails to develop, as well as embryonic patterning mutants. Only one gene has been cloned from this class, *EMB30* (Table 2). However, several untagged mutants from this class have been characterized. These include *twin* (Vernon and Meinke, 1994), *lec* (Meinke, 1992; Meinke *et al.*, 1994; West *et al.*, 1994), *sus1* and *sus3* (Schwartz *et al.*, 1994), and *raspberry2* (Yadegari *et al.*, 1994).

The physiological class constitutes a diverse group of mutants. Included are flowering time (early and late), epicuticular wax, wilty, high florescence, lesion mimic, hormone, and lipid mutants among others. Many genes have been cloned from this class including *AUX1, CTR1, HLS, CER1, CER2, CER3, FAD2, FAD3, HY3, HY4, LD, RCN1* and *CHL1* (Table 2). A number of untagged mutants, e.g., *cer6*, and *cer10* (McNevin *et al.*, 1993), *chl7* (LaBrie *et al.*, 1992), *cop8*, *cop10* and *cop11* (Wei *et al.*, 1994b), have also been reported.

Seedling-lethal mutants are those in which the seeds germinate but do not grow; instead seedlings eventually turn brown and die. The frequency of transformed lines placed in this class is very subject to environmental conditions (Table 1; Feldmann, 1991; Feldmann *et al.*, 1994). No mutants in this class have yet been characterized.

Reduced-fertility mutants include mutants which are as tall as wild-type but produce a vast deficiency of seeds. No genes have yet been published from this class; however, several tagged and untagged mutants have recently been characterized and genes are being isolated (Chaudhury *et al.*, 1994; He *et al.*, 1996; Peirson *et al.*, 1996).

Exceptional Segregants/Gametophytic Mutants

The transformants that segregate in an exceptional manner for a vast deficiency of Kan^R seedlings (e.g., 1 : 100 or 1 : 1 [R : S]; 9% of the total) have been tested through multiple generations of selfing and through crosses. Of 17 lines tested, seven continue to segregate in an exceptional manner (~ $1Kan^R$: $3Kan^S$; Feldmann *et al.*, 1997). In crosses with wild-type plants, two of these fail to transmit the Kan^R marker through the female while the other five transmit the Kan^R at a very low frequency through the male. However, none of these lines is specific for one sex as 1 : 1 (Kan^R : Kan^S) ratios are not observed in the reciprocal crosses but rather resemble ratios that are similar to those from selfed plants. The two lines that fail to transmit through the female possess 50% aborted ovules, indicating that these are gametophytic mutants. Characterization of these exceptional segregants is an excellent method for identifying genes in gametophytic growth and development. Also, the identification of a mutant exhibiting the exceptional segregation ratio greatly increases the probability that the affected gene is tagged.

Distribution of the Inserts in the Gene and Genome

There are numerous pieces of evidence that show T-DNA insertion is random in the gene and genome. When T-DNAs responsible for over three dozen visible

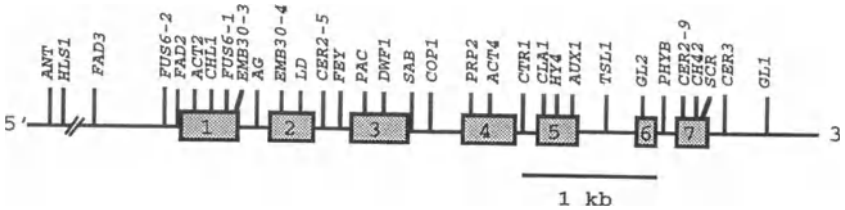

Figure 1. Distribution of T-DNAs in a generic *Arabidopsis* gene. Number of exons (open boxes) and gene length calculated from cloned T-DNA tagged genes. The average gene is 4.0 kb from the transcription start site to the polyadenylation signal and contains six introns and seven exons. Inserts are shown in the approximate location where they were found in the tagged gene

mutations are characterized, we find them in exons, introns, and 5′ and 3′ regions (Fig. 1). Second, the ~ 70 T-DNAs that have been placed on the genetic map tend to map randomly (Fig. 2; Franzmann *et al.*, 1995; our unpublished results). Third, from screening the first 14 000 transformants, no apparent hot spots have been

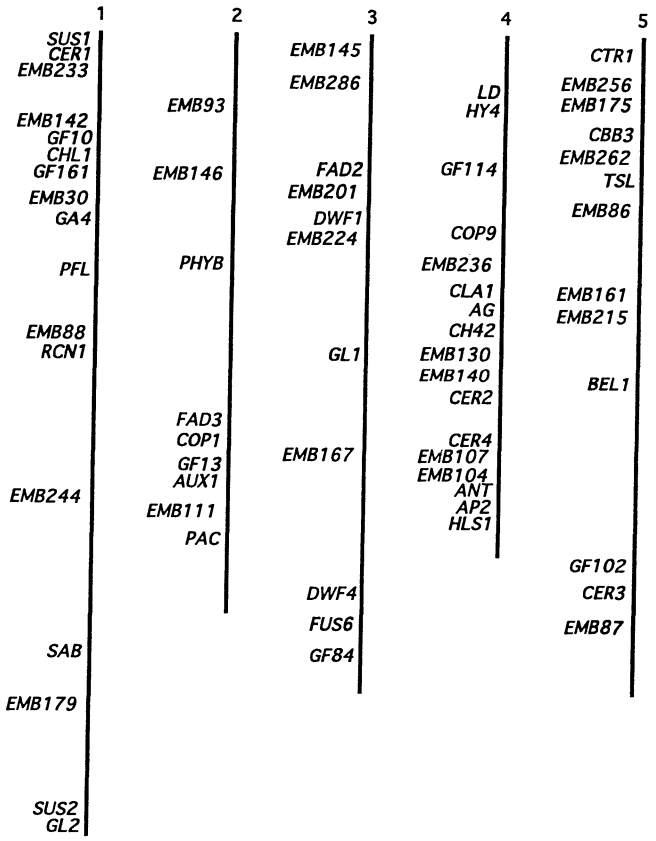

Figure 2. Distribution of T-DNA insertion sites on the five chromosomes of *Arabidopsis*. EMB lines are from Franzmann *et al.*, 1995, and D. Meinke, personal communication)

observed. For example, we have not found more than three tagged alleles of any of the seven dwarf loci. *AGAMOUS*, which represents > 5.5 kb of genomic DNA, has only been mutated twice in the transformed population (Yanofsky *et al.*, 1990; our unpublished observation). Finally, for several well-characterized phenotypes, e.g., epicuticular wax and dwarf mutants, we have tagged close to 50% of the known loci that contribute to each of these phenotypes (Feldmann, 1991; Forsthoefel *et al.*, 1992; McNevin *et al.*, 1993; unpublished results); this is a result of screening only 14 000 lines (21 000 inserts). Tight linkage has been shown for four of the nine *cer* loci that belong to phenotypic groups I and II (McNevin *et al.*, 1993); three of these genes have already been cloned (Table 2). Of the seven brassinolide-responsive dwarf loci that have been identified, we have tagged alleles of at least three (our unpublished results). This is also the trend for ethylene-affected mutants, where J. Ecker and his colleagues have shown that, of eight loci with recessive alleles, three are tagged (Kieber *et al.*, 1993; Lehman *et al.*, 1996; J. Ecker, personal communication). These combined data, the randomness of the inserts within the gene and genome, and the tagging of nearly 50% of the genes for a number of phenotypes, strongly supports our assertion that the T-DNAs insert randomly. This does not discount preferential integration into transcribed regions or other mechanisms that prevent insertion into localized regions of the genome.

Putative Somaclonal Variants and Untagged Mutants

Mutants isolated from transformed populations that do not cosegregate with the selectable marker in the T-DNA can be of significant importance to an investigator in choosing between alternative approaches to mutant screening and gene cloning. There are several ways in which these untagged mutants can arise in transformed populations, including mutations resulting from tissue culture procedures, abortive integration, spontaneous mutation, and possibly mutagenesis due to the presence of *Agrobacterium* or T-DNA. On the positive side, if T-DNAs insert randomly and if the vast majority of untagged mutant alleles are due to abortive integration events (leaving footprints) then many novel mutants will be isolated; these in themselves may be valuable to plant biologists attempting to saturate a particular pathway.

Somaclonal Variation

In tissue culture transformation systems, mutants can result from long exposures to exogenous hormones, creating 'somaclonal variants'. Somaclonal mutants result from many different types of mutagenic events including, but not limited to, point mutations, chromosomal rearrangements and heteroploidy (Lee and Phillips, 1988). If the percentage of somaclonal mutants was high it may actually mask the tagged mutants in the population. However, while somaclonal variation can account for some of the untagged mutants observed among tissue culture-derived

transformants (Valvekens and Van Montagu, 1990), it cannot explain the untagged mutants found in transformants generated with non-tissue culture transformation procedures (Feldmann, 1991).

Even in these latter transformants as many as 60–65% of the mutants fail to cosegregate with the kanamycin-resistance marker in the T-DNA. For example, Castle *et al.* (1993) performed cosegregation analyses on 115 of 178 embryo-defective mutants isolated and observed that only 41 (36%) of these mutants were tightly linked to a Kan^R marker in the genome. Similar results were observed by McNevin *et al.* (1993) for epicuticular wax mutants and for dwarfs (our unpublished results). Interestingly, Castle *et al.* (1993) identified one embryo-lethal line that contained a truncated T-DNA lacking the kanamycin-resistance marker. However, this may be the exception rather than the rule as sequencing of nine other mutant alleles from the T-DNA population, which failed to cosegregate with the kanamycin-resistance marker, showed only base substitutions or deletions (see below).

Abortive Integration

It has been speculated that many of these mutants are due to abortive T-DNA integration events. In order to understand how this might happen it is necessary to understand what happens during T-DNA integration and to examine the sequence of some of the untagged mutants (Table 3). This summary will be limited to what is hypothesized to happen to the T-DNA in the plant cell. Tinland and Hohn (1995) and Tinland (1996) hypothesize that T-DNA enters the plant cell as a T-complex composed of a VirD2 protein at the 5′ end of the T-DNA (RB) acting as a pilot. This T-complex enters the nucleus through a nuclear pore. The left border finds homologies to the plant DNA at a point where single stranded DNA is exposed (e.g., transcribed regions). The displaced plant DNA and the 3′ overhanging DNA of the T-DNA are subjected to exonuclease activity. The VirD2 protein on the 5′ end of the T-DNA complex searches for microhomologies adjacent to the LB attachment site. The VirD2 complex is annealed to the plant DNA strand. The 3′ end of the annealed T-strand primes the synthesis of plant DNA. The other strand of plant DNA is extended by repair replication to copy the T-DNA while the nucleotides up to the microhomology where the RB attached are degraded. This leads to deletion of a small stretch of the target DNA. The phosphotyrosine bond binding the VirD2 protein, at the 5′ end of the T-DNA, is exchanged for a phosphodiester bond and T-DNA integration is complete. Two general conclusions pertaining to T-DNA integration from this model are important for this discussion. First, T-DNA integration is not site specific and does not require long stretches of homology for integration. Thus at the 3′ end (LB) the mode of integration appears to be via illegitimate recombination. Second, integration of the T-DNA leads to small deletions at the site of insertion. The deletion can be as short as seven nucleotides (Yanofsky *et al.*, 1990) and as long as 73 nucleotides (Mayerhofer *et al.*, 1991). As such, if insertion were aborted after both the 5′ and 3′ ends anneal, small deletions will be created.

An examination of the sequences of nine untagged alleles from the transformed population shows that four fit exactly into this model while another had a longer deletion (1.2 kb) and two had shorter deletions (1 and 2 bp; Table 3). The final two possess single base-pair changes. From this limited set of data, we speculate that few untagged mutations are the result of truncated T-DNAs lacking the selectable marker, while most are a result of some type of base-pair-deleting mechanism.

Other Mechanisms to Account for Untagged Mutants

While the untagged alleles with short or even long deletions may be the result of abortive T-DNA integration, this would not seem to be the cause of the single base changes (Table 3). The base substitutions are probably the result of a combination of spontaneous mutation (given the large number of transformants that are screened) and mutations induced by *Agrobacterium* (by some unknown mechanism). In fact, in a successful transformation experiment, a small percentage of the infected plants (T_1) will contain an albino sector similar to what is observed after EMS mutagenesis. Additionally, on at least one occasion we have found a mutant in the T_4 generation which could not be found in the T_3 families, suggesting that a new mutation had arisen. These observations suggest the possibility that the presence of *Agrobacterium* or T-DNA itself induces a DNA repair system which in turn causes point mutations. This latter possibility is of concern because of the large percentage of T_3 families identified that segregate for a deficiency of mutants (e.g., 1 : 10 mutant wild-type; Feldmann, 1991). The mutations in these lines are not due to a functional T-DNA insert.

While T-DNA insertion mutagenesis strategies can result in untagged mutants, it should be noted that many nascent mutants have been isolated from these populations. Even with extensive screening, mutations in several genes identified in the T-DNA population have still not been recovered from ethylmethanesulphonate (EMS) mutagenized populations (e.g., LaBrie *et al.*, 1992; Berger *et al.*, 1996). This adds evidence to our assertion that T-DNAs insert randomly in the genome (Forsthoefel *et al.*, 1992).

From Gene to Phenotype with T-DNA Transformants

Large numbers of gene sequences are being derived from plant species, including corn, rice and *Arabidopsis*, for which nothing is known about the phenotype to which they contribute. The sequences have been derived by: (1) random sequencing of cDNAs; (2) homology to heterologous probes; (3) homology to phenotype-specific genes; or (4) comparative techniques (e.g., subtractive hybridization and differential display). Comparison of these sequences with gene sequences of known function may provide the first clue about their role in normal plant growth and development. In an attempt to further elucidate a function for a gene of interest, researchers often perform a series of expression and localization studies and

may even conduct overexpression or antisense inhibition experiments. The results from these experiments are, more often than not, inconclusive. A mutant phenotype for the sequence of interest could provide considerably more insight into the *in-vivo* function of the protein in the plant.

A reverse genetics procedure that enables the identification of an insertion mutant for a sequence of interest was developed for the T-DNA-generated populations of *Arabidopsis* by McKinney *et al.* (1995). Primers from the T-DNA border regions and a sequence of interest are used to identify a PCR product in DNAs from large pools of segregating transformants (McKinney *et al.*, 1995; Winkler and Feldmann, 1996). What is required to make this an efficient screen for any sequence of interest is a large population of T-DNA-generated transformants, random insertion, and pooled DNAs. All three of these criteria have been met. There is already a sufficiently large population of transformants to begin a reverse genetics approach and over the next 2–3 years the size should approach what is needed for saturation. In addition, DNA has already been extracted from pools of transformants and is available to the research community through the *Arabidopsis* Biological Resource Centers. Finally, as described above, T-DNAs appear to be distributed randomly in the gene and the genome.

In preliminary experiments with DNA isolated from 5300 lines, McKinney *et al.* (1995) screened DNA from pools of 100 transformants with primers homologous to the left or right border in combination with degenerate reverse or forward primers made from conserved regions of 10 actin genes. Two actin null mutants were observed: *act2-1* and *act4-1*. The T-DNA insert in *act2-1* was immediately 5′ of the ATG start site and for *act4-1* the insert was in the third exon (McKinney *et al.*, 1995). The insert in *act2-1* contained two right border–plant junctions while *act4-1* had two left border–plant junctions.

Conclusions

T-DNA insertion mutagenesis has found wide popularity in *Arabidopsis* because of the genetic and biological features of this species and the ease of cloning tagged genes. More than 20 000 T-DNA transformants have been generated in this species; this population contains 30 000 inserts. More than 4000 visible mutants have been identified in this population which fall into a variety of phenotypic classes (Table 1 and data not shown). Genetic and molecular analyses indicate that 35–40% of the mutants are due to a T-DNA insert. Given random insertion (Figs 1 and 2) and a genome of 120 Mbp there is already a > 65% probability of an insert in any average gene of *Arabidopsis*. More than 40 genes have already been cloned and characterized (Table 2 and our unpublished results).

Untagged mutants (Table 3) appear to be primarily a result of somaclonal variation (in tissue culture-derived transformants) and abortive integration events. Spontaneous mutation and other mutagenic processes probably come into play as well, but their importance in generating these mutants has yet to be ascertained.

A PCR-based reverse genetics developed for this species will not only avoid the untagged mutants but will give us information about the 90–95% of genes for which there will be no visibly altered phenotype. This strategy will also rapidly increase the number of genes associated with a phenotype such that our knowledge base, pertaining to T-DNA tagging in *Arabidopsis*, will expand exponentially.

As many of the genes that have been identified in *Arabidopsis* are homologous to genes in agronomic species, this is a powerful approach for identifying agronomically important genes for crop plants.

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27. Phenotypic Variation Between Transgenic Plants: What is Making Gene Expression Unpredictable?

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Phenotypic Stability and Variability

From the very beginnings of genetic engineering, researchers have questioned whether the genes that were being transformed into plants would be expressed as predictably as those that had evolved there. In one of the most extensive of the early studies on the inheritance of newly introduced traits, Müller *et al.* (1987) reported that the rate of transgene inactivation was comparable to rates of spontaneous mutation for a naturally evolved gene. To measure this, they introduced the coding region for neomycin phosphotransferase *II* (*npt*) driven by the nopaline synthase promoter (nos_{pr}) into tobacco. When homozygous transformants with a single copy of the nos_{pr}-*npt* gene were back-crossed to untransformed plants, the frequencies of nos_{pr}-*npt* gene inactivation in the progenies of two transgenic lines were 6/45 000 and 25/45 000. Unfortunately, none of those plants that appeared to have lost resistance to kanamycin could be rescued before they died. As a result, it was not possible to determine whether the loss of the trait was due to mutational inactivation of the gene or to epigenetic suppression of the phenotype. In a more recent study, Dehio and Schell (1993) produced morphologically altered *Arabidopsis* plants by introducing a single copy of the *Agrobacterium rhizogenes rol*A gene. They then screened the progeny of homozygous plants for individuals that reverted to normal. None was found within a sampling of 10 000 progeny. In fact, reversion to a wild-type appearance was only seen among the M_2 progeny of plants treated with the chemical mutagen ethylmethanesulphonate (EMS). Even here, only 12 apparently normal plants were observed from a population of 360 000 M_2 plants and none from 45 000 M_1 plants.

These types of studies showed that most transgenes displayed the heritability and consistent expression expected of them by their builders based on their understanding of genetics at that time. Nevertheless, in some cases, the same constructs in the same experiments produced primary transformants that consistently showed either variegation (unexpected variation in gene expression between otherwise identical cell types) or complete loss of transgene expression throughout the organism in the first or second generation. Not only did these problems persist during the life of the plants, but they were often transmitted to successive generations as well. This heritable deviation from the anticipated level of expression is due primarily to an epigenetic process called silencing.

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Phenotypic Variability of Transgene Expression

There have been a number of well-documented examples in genetic systems like *Drosophila* that show that expression of a locus can be highly variable or silenced when it is translocated from its normal site on a chromosome to positions near telomeres and centromeres (Ephrussi and Sutton, 1944; Rushlow *et al.*, 1984). At first, it was thought that transgenes showing similar inconsistency in expression were similarly affected by the unusual forms of chromatin found near their new locations. Before the widespread nature of silencing became apparent, many of the transformants with little or no transgene expression were probably discarded so that the investigator's attention could focus on more successfully functioning lines. Experiments to study phenotypic instability and gene silencing were only begun when it was demonstrated that these aberrations could be generated so predictably (Matzke *et al.*, 1989, 1993) or so dramatically (Napoli *et al.*, 1990; van der Krol *et al.*, 1990a) that the results could not be dismissed as mistakes. Despite numerous investigations, there is still no single, persuasive explanation for the variability in expression that has been seen in some transformed individuals but most of the data can be explained by one of two mechanisms operating at different levels of gene expression: transcriptional and post-transcriptional (Matzke and Matzke, 1995; Meyer, 1995). As is often the case in science, some of the early studies failed to investigate processes whose importance came to be recognized much later. Thus, the experiments needed to differentiate between the two contrasting levels of silencing have not always been done, so that those cases must be analyzed cautiously.

Transcriptionally silent genes either produce less RNA than expected, or none at all, or produce it in some cells, but not others. In a few cases, transcription ceases when a second copy or part of a copy of the transgene is introduced elsewhere in the genome (Matzke *et al.*, 1989). In other cases, the level of transcription is correlated (Matzke *et al.*, 1994; Park *et al.*, 1996), or presumed to be correlated, with the proximity of the transgene to normally silent regions of the chromosome. Since transgenes can, in a few cases, be reactivated by growing cells with 5-methylcytidine, it has been argued that integration into or near heavily methylated regions of the chromosome leads to reduced transcription through transgene methylation (Hepburn *et al.*, 1983). However, only one study (Pröls and Meyer, 1992) has actually demonstrated that methylation of the new gene is similar to that found at adjacent chromosomal sequences. It is worth keeping in mind that variegation and silencing occurs in *Drosophila* (Henikoff, 1994) which contains very little detectable 5-methylcytidine (Urieli-Shoval *et al.*, 1982), and that a process similar to these, called 'quelling', occurs in *Neurospora* mutants lacking cytosine methylation (Cogoni *et al.*, 1996).

The second type of silencing acts at the post-transcriptional level. It appears to be dependent on both the location of the gene in the genome and on its initial expression there. In these cases, an introduced gene is expressed initially in the transformant or its progeny, but then shuts down 2–7 weeks after germination as if the amounts of messenger or protein made from the transgene have exceeded

the capabilities of the system (de Carvalho *et al.*, 1992; Dehio and Schell, 1994; de Carvalho Niebel *et al.*, 1995; Schmülling and Röhrig, 1995). In several cases, the phenotypically silenced genes were still being transcribed in the nucleus, yet little of this RNA accumulated in the cytoplasm (de Carvalho *et al.*, 1992; de Carvalho Niebel *et al.*, 1995; Van Blokland *et al.*, 1994; English *et al.*, 1996; Park *et al.*, 1996). In some cases, this shut-down correlated with increased transgene methylation (Meyer and Heidmann, 1994; Elomaa *et al.*, 1995; English *et al.*, 1996; Park *et al.*, 1996) while in others it did not (Goring *et al.*, 1991; Hart *et al.*, 1992; Fujiwara *et al.*, 1993; Mittelsten Scheid *et al.*, 1994; Schmülling and Röhrig, 1995). However, the failure to find methylated bases at one or more sites does not mean that other, more crucial regions of a gene, have not been modified. Conversely, finding methylation at some sites does not necessarily mean they are responsible for blocking transcription. For instance, Enver *et al.* (1988) showed nearly a decade ago that reduced gene expression, however it is caused initially, precedes rather than follows methylation. Methylation may merely provide a more persistent form of suppression for genes that are no longer needed during the life of the organism.

The following sections of this chapter will introduce additional examples of transcriptional and post-transcriptional silencing. It may turn out that each results from unrelated processes, but as more studies are completed it appears that the phenomena may differ more in the degree and timing of appearance than in the mechanisms of their implementation (English *et al.*, 1996; Park *et al.*, 1996). We will therefore evaluate unifying models that might be able to account for the disparate results.

A Brief Synopsis of Transcriptional Silencing

In general, the more easily one can monitor slight changes in gene expression, the more likely one finds partial or complete silencing. The best of the current systems for visualizing spatial differences in the magnitude of gene expression uses genes that alter flower pigmentation. For example, the maize dihydroflavonol 4-reductase encoded by the A1 gene of maize (Reddy *et al.*, 1987) converts dihydrokaempferol into leucopelargonidin, leading to accumulation of brick-red pelargonidin derivatives (Meyer *et al.*, 1987). When this gene was introduced into white-flowered petunias under the control of the 35S promoter of cauliflower mosaic virus (CaMV) some plants produced completely red flowers. Most, though, produced variegated red and white flowers (Meyer *et al.*, 1987; Linn *et al.*, 1990). As in all cases of phenotypic variation discussed in this chapter, the white cells are genetically identical to the red cells, but epigenetically distinct and so fail to express the new gene product adequately.

Why should two plants, each with the same parentage and each transformed with the same construct, show such differences in phenotypic stability? Since the frequency of silencing (approximately 10%) is much higher than the frequency of *deletions or nucleotide changes*, and in many cases is reversible by outcrossing,

there has never been wide acceptance for explanations based upon transgene mutagenesis at the time of integration. Instead, attention has focused on post-replicative modifications of chromosomal DNA that are almost as effective as deletions at altering transcriptional processes. Like many multicellular eukaryotes, portions of the plant genome are methylated (Gruenbaum *et al.*, 1982), and the degree of methylation, especially of promoter sequences, correlates with a reduction of gene expression (Hepburn *et al.*, 1983). One possibility, therefore, is that the difference between variegating and phenotypically stable transformants depends on whether the gene is integrated near heavily methylated regions of the chromosome where it would be frequently modified by DNA methylases working nearby. Investigation into the degree of modification of the *A1* cDNA gene and 35S promoter (Meyer *et al.*, 1992; Meyer and Heidmann, 1994) has shown that the decrease in *A1* gene activity in red and white tissues correlates with the degree of hypomethylation. Linn *et al.* (1990) showed that methylation of two CCGG sequences at –315 and +11 within the 35S promoter was especially correlated with reduction of mRNA accumulation and white or variegated phenotypes.

While these studies were under way, a very different set of constructs was providing similar correlations between silencing and transgene methylation. Matzke *et al.* (1989) had transformed tobacco with a T-DNA containing a *nos*$_{pr}$-*npt* gene. They then wished to introduce a new T-DNA by selecting for expression of a *nos*$_{pr}$-hygromycin phosphotransferase (*hpt*). In each case, though, they found that the introduction of the new gene silenced the *nos*$_{pr}$-*npt* gene that had been introduced previously. This suppression was reversible: crossing the silent locus into an untransformed background restored gene expression. Furthermore, silenced genes that appeared to be methylated at position –222 within their promoter became partially or completely demethylated when expression was restored.

Other pairs of constructs were found that behaved in a very similar way. Neuhuber *et al.* (1994) characterized four independent transgenic tobacco lines containing chimaeric genes that used independent copies of the 35S promoter to express genes for hygromycin resistance and chloramphenicol acetyltransferase (*cat*). Each of these plants was then retransformed with a construct containing the octopine synthase (*ocs*) gene driven by a 35S promoter. In two of the four lineages, the *hpt* and/or *cat* genes became completely or partially inactive in about 4–10% of the regenerated plants containing the 35S-*ocs* cassette. In some cases, silencing did not occur immediately. Instead, the *hpt/cat* locus was expressed at germination and then became inactivated within 6–8 weeks. On the whole, the propensity to silence increased with gene dosage. Thus, the *hpt/cat* locus with its two copies of the 35S$_{pr}$ was prone to silencing even in the absence of the superinfecting T-DNA, but was suppressed more thoroughly after introduction of the second locus. Moreover, homozygous progenies were more likely to become silenced than hemizygous ones in both the initial and superinfected transformants. Although methylation patterns differed from plant to plant, there was a correlation between the reduction in transcription of the inactivated genes in isolated nuclei and methylation of their promoters.

A Brief Synopsis of Post-transcriptional Silencing

The phenotype associated with the genes described in the previous section closely matched their transcriptional activity. While post-transcriptional silencing may produce the same phenotypes as those examples, it appears to result from a very different process. In all of the cases where it has been investigated, the silenced gene is still being transcribed, but the primary transcripts are either not exported from the nucleus, or are quickly destroyed once they enter the cytoplasm (Van Blokland *et al.*, 1994). In these cases, the phenotypically silenced gene also silences endogenous genes homologous to it. For example, β-1, 3-glucanase genes with 81% DNA sequence homology with a transgene showed reduced expression while isozymes with about 63% DNA sequence homology were not co-suppressed (de Carvalho Niebel *et al.*, 1995). Similarly, petunias transformed with new copies of either chalcone synthase (*chs*) or dihydroflavonol reductase (*dfr*) genes suppressed both themselves and their homologues so that genetically wild-type flowers failed to produce normal levels of messenger and normal amounts of pigment (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990a).

The distribution of white and purple cells in these petunia flowers varies throughout the organ, but does not follow known cell lineages. Nevertheless, the star and 'Russian-dancer' patterns within the flower show a degree of regularity associated only with developmental processes. Hart *et al.* (1992) demonstrated that development also influences the co-suppression of a chitinase gene in transgenic tobacco plants. In this case, the immature or later developing (top) leaves had lower levels of chitinase than the maturer or earlier developing (lower) ones. Co-suppression of genes for a β-1, 3-glucanase (de Carvalho *et al.*, 1992), an *S*-adenosyl-L-methionine synthase (Boerjan *et al.*, 1994), and a nitrite reductase (Dorlhac de Borne *et al.*, 1994; Vaucheret *et al.*, 1995) also follows the same spatial or developmental gradient.

Gene Inactivation can be Exasperated by Tissue-culture Conditions

Many of the studies described in this chapter have used plants regenerated from cells of a single parent. Even untransformed plants derived from this process often show differences under the same growth conditions. The source for this somaclonal variation has not been explained. It has been proposed that animal cells that start out identical may change as stochastic processes activate or suppress genes during critical periods of development (Elliot *et al.*, 1995). Studies on plants as different from each other as carrot (Vergara *et al.*, 1990), tobacco (Anderson *et al.*, 1990), and rice (Müller *et al.*, 1990) have shown that tissue-culture conditions can alter either replicative or post-replicative processes responsible for maintaining faithful transfer of genetic information to the next mitotic generation. The longer these plants are exposed to hormones and other chemicals that are used to stimulate *in-vitro* growth, the greater the increase in DNA methylation and in heritable or non-heritable somaclonal changes in their

phenotypes(for a review, see Phillips *et al.*, 1994). Application of new methods of plant transformation which do not require tissue culture such as *Agrobacterium*- or electroporation-mediated gene transfer to growing plants (Bechtold *et al.*, 1993; Katavic *et al.*, 1994; Chowrira *et al.*, 1995) might be able to reduce the magnitude, but not necessarily the cause of silencing. In order to understand what that cause might be, we must first identify the independent components of gene expression that might be susceptible.

Normal Determinants of Transcriptional Uniformity

If one approaches a genetic engineering project pragmatically, the process of assembling and inserting a gene so that it will be expressed in a new host appears very straightforward. A promoter must contain *cis*-acting elements where TATA-binding proteins like TF ('transcription factor') IID and various enhancer-binding proteins bind either to provide a landing platform for RNA polymerase II, or to modify bending, torsion, or chromatin structure over the entire region of the gene that RNA polymerase II must transcribe (reviewed in Zawel and Reinberg, 1995).

Once transcription begins, it is expected to continue until the primary transcript is complete and terminates. No one has identified DNA sequences that determine the termination of transcription in plants, but based on studies of animal genes these terminators may lie 1–2 kb beyond the last base retained in the messenger (Citron *et al.*, 1984; Iwamoto *et al.*, 1986). This primary transcript is then processed so that a new 3′-end is added 10–30 nucleotides downstream of a poly(A) addition site; 3′-end formation is thought to occur the same way in plants. Deletion studies have shown that genes without a poly(A) addition site or terminator are poorly expressed (Ingelbrecht *et al.*, 1989). Different DNA frag0ments isolated from the 3′-ends of both T-DNA and plant genes can increase this expression up to 60-fold depending on which is used. None of these fragments greatly alters the end of the mRNA so it is presumed this range of expression is due to sequence-determined differences in either termination efficiency or primary transcript processing. It must be remembered, though, that the investigator chose each 3′-end only by the availability of useful restriction sites so that important sequences lying distal to those might have been omitted. It is possible, therefore, that these terminators differ little in their original genomic context.

Genomic Context may be Influencing Gene Expression

In addition to 3′-end formation, the completed primary transcript must undergo several different steps in processing before being exported to the cytoplasm. First, a cleavage reaction must generate a defined 3′-end, and then a chain of adenosines must be added so that the finished message can be exported. If the gene contains

introns, these must be removed. One of the less expected discoveries in this field over the past few years has been that these processing steps occur along a well-defined trail leading from the gene itself to the nuclear periphery (Carter *et al.*, 1993; Xing *et al.*, 1993). It is as if genes must lie near specific nuclear channels, that we will refer to later as *nuclear processing tracts*, for 3′- and intron-processing, and mRNA export to proceed naturally. We presume these tracts are anchored in some way to sites near the genes. It may be that some of the discrepancies in RNA splicing or mRNA synthesis that have been noted during transient expression studies are due in part to the accessibility of genes to these tracts. Adami and Babiss (1991), for example, found that two identical copies of an adenovirus gene introduced independently into the same cell were spliced differently. They suggested that the choice of splice sites might be determined by the location of the template in the nucleus. In quite different experiments, Ingelbrecht *et al.* (1989) found that the efficiency of some terminators measured in transient assays decreased after they had integrated stably in a chromosome. One explanation for this result can be that the integrated genes had more restricted access than freely diffusing genes to enzymes or export channels associated with nuclear processing tracts.

The degree of tract organization within the nucleus would have to be considerable to handle the continual changes in transcription of the thousands of genes scattered over many chromosomes. Yet in fact, the nuclear matrix in both *Drosophila* (Manuelidis, 1990) and plant cells (Leitch *et al.*, 1991) has been shown to be highly organized. At least a small part of this order comes from the attachment of chromosomes to portions of the nuclear membrane and the nucleoplasm by means of elements called *MAR* (matrix attachment region) or *SAR* (nuclear scaffold attachment regions) sequences. Transgenes linked to such attachment points are expressed better than those that are not (see Spiker and Thompson (1996) for a critical analysis of these elements and their use), yet MAR and SAR sequences appear to have little effect on the variability of expression that is due to position effects. A second class of modulatory elements is called an *insulator.* These shield some genes from the effects of nearby enhancers (Cai and Levine, 1995). Most recently, a third type of element called an *LCR* (locus control region) has been described that is thought to ensure that proteins that assemble DNA into open chromatin configurations out-compete those that assemble genomic sequences into heterochromatin (Festenstein *et al.*, 1996). These sequences may lie as close as 500–1500 bases downstream from the poly(A) addition site (Lang *et al.*, 1991). Unlike insulators and possibly some MAR sequences, LCRs appear to reduce position effects significantly. None of these sequences is yet well defined and further studies may show they are interdependent or possibly even subsets of a single class of chromatin organizers. In any event, we believe SAR, LCR, and insulator sequences are involved in ensuring that fluctuations in activities of transcription factors, chromatin components, and methylases due to changes in temperature or precursor availability do not change the outcome of developmental pathways.

What Happens when Genomic Context is Changed?

Gene expression is normally canalized at every possible level so that all cells following the same developmental programme ultimately produce the same phenotype (Waddington and Robertson, 1966). These canalization processes have evolved to compensate for stochastic fluctuations in the amounts, assembly, and activities of enzymes and macromolecules resulting from fluctuations in ambient temperature or in the availability of basic chemical precursors. Mutations that disrupt these processes can reduce the number of cells in any given individual that follow a genetic programme faithfully. For example, chromosomal rearrangements in loci lying 15 kb away from the structural gene for xanthine dehydrogenase (*XDH*) can greatly reduce levels of *XDH* messenger in *Drosophila*. Histochemical analyses of the enzyme in these mutants has revealed that tissues show a mosaic of expressing and non-expressing cells instead of a uniform pattern (Rushlow *et al.*, 1984). These experiments demonstrate that, in this case, the mutation has not altered the magnitude or specificity of expression, but rather the frequency with which the programme for expression is followed. Translocations such as the one that produced this effect juxtapose transcribed genes next to untranscribed regions of the genome. It is believed that the heterochromatin of the latter region interferes with the assembly of euchromatin around the gene so that its regulatory sequences will be less accessible to transcription factors.

It has been suggested that chromatin structure can be influenced not only by the state of chromatin at adjacent loci, but also by chromatin *in trans*. This effect has been used to explain the properties of an allele of the *b* locus of maize (Patterson *et al.*, 1993). While most alleles of this gene segregate in a classical fashion, one called *B′* has been isolated that appears to imprint its defect on normal alleles in heterozygous plants. The primary change producing *B′* is still not known, but it does not entail base changes within the gene, changes in methylation within a 9 kb region encompassing the gene, or DNA rearrangements within a 24 kb region of the locus. Nevertheless, the mutant allele accumulates less nuclear RNA than its wild-type counterpart, and is able to suppress transcription of alleles on the homologous chromosome. The ability of a mutation to imprint an allele *in trans* to behave abnormally is called paramutation. It persists even after the affected allele has been crossed into a new background. Patterson *et al.* (1993) have suggested *B′* promotes abnormal chromatinization in its allelic partner. This then acts as a template to direct the chromatinization of the corresponding region of allelic sequences. Additional evidence for just this sort of allele-directed chromatin reorganization has come from studies of DNAse-sensitive regions of yeast genes (Keeney and Kleckner, 1996). As will be seen, some transgenes exhibit many of the properties of paramutations and could be explained in the same way.

Have Genetic Engineers Left Out Something Essential from their Chimaeric Genes?

In the majority of cases, transgenes are patchwork structures built from bits and pieces of several different genes. One gene may be used to provide a promoter in-

cluding essential transcriptional regulatory elements and the start of the transcription, while another gene is dissected to provide an intron. Both elements are then joined to the coding region of a third gene, and this is finally linked to the poly(A) addition site and transcriptional terminator of a fourth gene. Most of the interchangeable cassettes are defined as much by the convenience of their restriction sites as by the functions of the sequences. While the burgeoning number of transgenic plants shows these sequences perform well most of the time, the occasional case of silencing raises the nagging possibility that something is still missing.

The diversity of constructs showing silencing does not indicate that the phenotypic variability observed is due simply to the use of one particularly defective transcriptional element (see Table 1 for summary and the following text for

Table 1. Factors that could contribute to transgene silencing

Factor	Contribution	Reference*	Comments
Specific promoter	No	Matzke *et al.*, 1994	Most constructs use 35S promoter but there are examples of silencing using other promoters.
Specific intron	No	van der Krol, 1990a	Transgene inactivation is also reported with intron-less trangenes.
Transcibred sequence	In some cases	English *et al.* 1996	Silencing can be dependent on homology within coding region
Copy number	In some cases	van der Krol, 1990a	But silencing is reported with a single copy of transgene
Developmental conditions	Yes	Meyer *et al.*, 1992	Transgene inactivation appears at certain stages of plant development.
Environmental conditions	Yes	Hart *et al.*, 1992	Specific environmental influences have not been defined, but light and temperature may influence the degree of silencing.
Specific host	No	Meyer *et al.*, 1987; Bender and Fink, 1995	Silencing is seen in different transgenic plant species.
Specific tissue	No	Linn *et al.*, 1990; Matzke *et al.*, 1989	Gene inactivation is shown in different plant tissues.
Expression level	In some cases	Van Blokland *et al.*, 1994	Silencing is observed in plants with moderate or high levels of transcription.
Post-transcriptional	Yes	de Carvalho *et al.*, 1992	Most co-suppressed genes are still transcribed in nucleus.
Proximity to heterochromatin	Possibly	Park *et al.*, 1996	But no correlation yet established in plants between expression and specific location on chromosome.
Homozygosity	In some cases	de Carvalho Niebel *et al.*, 1995	But expression-dependent silencing occurs in both homozygous and hemizygous transformants.
Induced mutation	None found	Pröls and Meyer, 1992	None has been found in transgenes which have been cloned from transformant and sequenced.

*Additional references in text.

explanation and elaboration). While a large fraction of the examples of silencing used the 35S promoter (e.g., Napoli *et al.*, 1990; Goring *et al.*, 1991; de Carvalho *et al.*, 1992; Meyer *et al.*, 1993), this may be coincidental, since this is the most widely used promoter in plant research. Moreover, there are additional examples in which researchers have used the weaker nopaline synthase promoter (Matzke *et al.*, 1989; Fujiwara *et al.*, 1993) or the developmentally regulated chalcone synthase promoter (Van der Krol *et al.*, 1990a) and observed similar variations of activity.

There is less reason to believe that silencing is caused by failures of specific transcriptional terminators since so many have been used in building transgenes. Examples of silencing have occurred with genes ending with sequences from CaMV (Goring *et al.*, 1991), T-DNA genes *rol*C (Schmülling and Röhrig, 1995) or *nos* (Fujiwara *et al.*, 1993), and chalcone synthase (van der Krol *et al.*, 1990a). Nor does it appear that specific introns promote silencing to any significant degree (e.g., van der Krol *et al.*, 1990a; de Carvalho *et al.*, 1992; Schmülling and Röhrig, 1995).

Having tentatively absolved virtually all of the obvious components of the gene, we have left ourselves with little to consider. At first glance, the coding region seems the least likely factor to affect silencing. None of the genes that have been silenced share extensive homology (e.g., nopaline synthase, chitinase, and β-glucuronidase are very different from each other in size and sequence), or a common origin (some of these genes evolved in plants, some in bacteria). Nevertheless, there are some lines of evidence that indicate that the transcribed part of the gene may play as great a role in silencing as the classical regulatory regions of the gene.

First, Van Blockland *et al.* (1994) found that a promoterless copy of chalcone synthase could silence or partially silence the endogenous copies in three out of 15 transformants. The residual levels of transcription of sense and antisense RNA that were detected did not correlate with the magnitude of co-suppression.

Second, many genes are transcribed normally in the nucleus even though no messenger RNA can be found in the cytoplasm. This was first shown by de Carvalho *et al.* (1992) with a transgenic tobacco line called T17 that contained a chimaeric β-glucanase gene. While most transgenics expressed the gene as expected from its copy number, this line produced high levels of messenger and enzyme in hemizygotes, but not in homozygotes or in haploid plants. Other lines behaving in a similar fashion have been isolated with the same β-glucanase construct (de Carvalho Niebel *et al.*, 1995). In some although not all cases, plants with one copy of the locus from T17, and one copy of one of these loci, were also silenced. These and subsequent studies (de Carvalho *et al.*, 1992; de Carvalho Niebel *et al.*, 1995) showed that there was no apparent block in nuclear transcription anywhere along the length of the gene although splicing was somewhat reduced and very little of the processed RNA accumulated. Reports of other genes behaving in a similar fashion quickly followed (Hart *et al.*, 1992; Van Blokland *et al.*, 1994; English *et al.*, 1996; Park *et al.*, 1996). These studies show that the failure to detect mRNA is not always due to the failure to initiate or elongate

specific transcriptional units. Instead, the failure appears to lie in part in processing the primary transcript or exporting it correctly to the cytoplasm.

The third line of evidence implicating the role of the transcribed or coding region in silencing is a recent experiment by Elomaa *et al.* (1995) who compared the expression of two chimaeric genes in petunia. Each gene used the same transcriptional control elements, was introduced in plants in the same way, and encoded the same enzyme, dihydroflavanol-4-reductase. However, one of the coding regions had been isolated from maize and had a G/C content of 60%, while the other came from the dicot *Gerbera* and was 39% G/C. Each should have produced uniformly orange-coloured flowers, but plants expressing the maize gene varied greatly in the levels of transcription of the mRNA, and in the penetrance of the phenotype. By contrast, plants containing the *Gerbera* gene showed levels of activity proportional to the number of copies of the transgene. The investigators suggested that the G/C-rich sequences within the maize coding region provided more targets for DNA methylases that could silence transcription of the gene.

English *et al.* (1996) have recently refined this experimental approach by using a tobacco line containing a 35S promoter : β-glucuronidase gene that is capable of post-transcriptionally silencing itself or other loci expressing β-glucuronidase. This suppressive locus (T4) even silenced β-glucuronidase genes brought in as part of an independently replicating viral genome. A series of deletions was then made within the viral copy of the β-glucuronidase gene and introduced into T4. Each infecting β-glucuronidase gene was silenced except for one deletion in the 3′-region of the open reading frame. This demonstrates that the silencing machinery, whatever it may be, was dependent on a sequence at the 3′-end of the transcribed region of this particular gene rather than any part of the transcriptional regulators.

Fundamental Generalizations about Gene Silencing

It is clear from this summary that we still do not known enough about gene expression in plants to account for all of the properties of silenced genes and variegated patterns of expression reported in the literature. On the other hand, we are beginning to amass sufficient data to reject some of the explanations that were considered very reasonable several years ago, and to suggest consideration of a few hitherto less obvious ones. These explanations must be consistent with the following seven generalizations that appear to apply to most of the work to date.

1. *Silencing is associated with the change in position of a gene* in the *chromosome.* Genes in their original locations appear less susceptible to silencing than those that have been (a) integrated at random into a new host, (b) translocated adjacent to heterochromatic or heavily methylated sites, or (c) provided with an altered genetic address as a result of deletion of neighbouring

sequences. Some genes are silent in their new location but do not affect the expression of other loci, while others can also silence homologous genes or transgenes (Matzke *et al.*, 1989; de Carvalho Niebel *et al.*, 1995; Park *et al.*, 1996). The silenced homologue may remain silent even when back-crossed to untransformed plants, or may regain partial activity (Park *et al.*, 1996).

2. *Transgenes may be particularly susceptible to silencing because they generally integrate through unconventional processes.* Silencing may be reproducible, but it is still not possible to predict when it will occur. One is only able to anticipate that a certain percentage of transformants containing the same construct will be expressed lower than the rest. Expression may be reduced by a factor of 2–5, or be below the level of detection. Several simple explanations have been offered for many of these atypical cases. For example, the transgenes may have integrated near negative transcriptional regulatory elements (e.g., see Peach and Velten, 1991) or transcriptionally inaccessible chromatin (e.g., see Fluhr *et al.*, 1986). Alternatively, integration may have created tandem or inverted repeats of either the transgenes or portions of the target that then attract methylases or proteins responsible for heterochromatin formation. These hypotheses do not appear to account for changes in post-transcriptional processes.
3. *The mechanism of silencing involves sequences within both transcribed and untranscribed portions of the gene.* This conclusion stems from studies on co-suppression presented in an earlier section of this chapter. As summarized there, not only is the transgene silenced in some plants, but so is any locus with significant homology to it. In many cases, silencing does not affect production of a nuclear RNA, only accumulation of cytoplasmic messenger. In at least one case, co-suppression clearly depended on a unique region in the transcribed 3′-end of the target gene (English *et al.*, 1996). Through analogous experiments, Cogoni *et al.* (1996) found silencing in *Neuropsora* required at least 132 bp within the transcribed region of the gene.
4. *Co-suppressive silencing is sequence specific.* The mechanism of silencing only affects highly homologous genes; it does not produce a catastrophic change in mRNA synthesis, turnover, or translation. As a result, the affected plants still appear normal and produce the same levels of major cellular proteins as unaffected plants (de Carvalho *et al.*, 1992). Imagining a process that provides this degree of specificity is the crux of our problem. It is equivalent to the challenge that faces biochemists working to identify how misfolded proteins are distinguished from correct ones and targeted for renaturation or destruction. Examination of the cases presented in this chapter suggests that a system able to distinguish between the abundance of virtually any transcript and thousands of others being synthesized at the same time will almost certainly have to use some form of highly individualized autogenous regulation, most likely employing the messenger or gene product of the affected locus.
5. *Silencing does not appear to depend solely on the accumulation of exceptional amounts of mRNA.* There has never been a simple correlation between either

transgene copy number or T-DNA configuration (that is, the number and orientation of T-DNAs at a given integration site), and the steady-state level of messenger from the integrated genes (Jones *et al.*, 1987; Peach and Velten, 1991). Several of the initial studies on silencing addressed the possibility that transgene transcription shut down if the level of expression exceeded an undefined threshold. It was apparent that some genes expressed to a high level in hemizygotes were silenced in homozygous plants (de Carvalho *et al.*, 1992; Dehio and Schell, 1994; Neuhuber *et al.*, 1994). This could indicate that doubling the copy number doubled the level of expression until nuclear export processes were overwhelmed. However, the level of nuclear RNA from phenotypically silenced genes did not always exceed that of either endogenous gene controls (Dehio and Schell, 1994; de Carvalho Niebel *et al.*, 1995; English *et al.*, 1996), or normally functioning transgenes (English *et al.*, 1996).

6. *The degree of silencing is sensitive to developmental or environmental conditions.* The pattern of pigmentation in flowers due to transgene silencing (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990a) shows the degree of silencing can vary from cell to cell similar to variegation of gene expression in *Drosophila.* In addition, the level of expression of a transgene can change depending on the culture conditions of the plants. For example, the flowers of petunia plants expressing *dfr* were more intensely red if formed early in the flowering season than if formed in later months (Meyer *et al.*, 1992). In another example, nearly 25% of the plants making a new chitinase were silenced if reared in closed containers while none of 96 showed silencing if openly reared in a greenhouse (Hart *et al.*, 1992).

   These same studies showed that silencing varies throughout the life of the plant. Chitinase activity in some plants, for instance, was high in the first leaves to mature and lower in the subsequent ones (Hart *et al.*, 1992). Once the specific genes were silenced in a leaf, their expression appeared to be fixed throughout the remainder of the life of the plant. Although de Carvalho *et al.* (1992) did not survey this particular developmental gradient, they did find that their plants had a functioning β-glucanase transgene at germination but that expression declined progressively with each week of growth.

7. *While some cases of silencing may be due to mutations in the gene sequence, there is no evidence that this accounts for the vast majority of the observations.* One class of silencing that has been carefully studied in fungi is called repeat-induced point mutations (RIP; Selker, 1990). This mechanism of mutation appears to be dependent on DNA–DNA interactions between identical genes. Nevertheless, in the few cases where silenced genes have been recloned and sequenced, no mutations have been found (Pröls and Meyer, 1992; Mittelsten Scheid *et al.*, 1994). Moreover, the frequency with which developmentally progressive silencing, post-transcriptional silencing, and meiotically reversed silencing occurs would require some form of directed mutagenesis operating at efficiencies that have not been seen even in bacteria (Foster and Cairns, 1992).

Current Models for Transgene Silencing

We have tried to present a summary of the representative types of experiments done to date on gene silencing in plants. The remainder of this chapter is meant to be controversial in hopes it will stimulate a fundamental reconsideration of the entire phenomenon and challenge the reader to test some of the alternative explanations available.

In our mind, there are currently three models for gene silencing that should be explored further. Each model focuses on how excess or aberrant transcripts are recognized, or how functional and silenced loci are distinguished by the cellular machinery. One was inherited from early studies of position effect in *Drosophila* and applied somewhat uncritically to the variations seen in plants. This model assumes that genes are inevitably silenced if they are introduced near heterochromatin. This presumably results because the new gene becomes incorporated into heterochromatic nucleosomes and so is inaccessible to transcription factors. In fact, there is not as much experimental support in plants for this assumption as might be supposed. Because fruit fly salivary-gland chromosomes are polytene, genes can be precisely mapped to specific bands visible in a light microscope. Neither the eu- and heterochromatin domains of plant chromosomes nor gene position with respect to them can be resolved so clearly. At best, one can show that the new genes lie on long or short chromosomal arms (Matzke *et al.*, 1994; Park *et al.*, 1996) but this resolution is not sufficient to say whether these sites are near transcribed loci or silent, intergenic regions. If these microscopic techniques can be improved, it should be possible in the future to determine how close heterochromatin must be to affect transgene activity in plants. It also remains to be seen whether this method of transcriptional interference affects genes *in trans.* and is able to act post-transcriptionally. Two additional models, one proposed recently by Lindbo *et al.* (1993) and English *et al.* (1996), and one introduced here, attempt to explain these other cases.

The two additional models are based to some extent on a pivotal study by Wassenegger *et al.* (1994) investigating replication of viroid RNA. Viroids are rather peculiar parasites of plants that do not encode any proteins, do not integrate into the host genome, and are not copied into DNA at any time in their life cycle. They normally replicate in the nucleus with the help of host enzymes. Wassenegger *et al.* fused a cDNA copy of a viroid to the 35S promoter and *nos* 3′-end and introduced this into tobacco by means of a T-DNA vector. During these studies they found that the integrated copy of the viroid became methylated whenever the free viroid began to replicate in its normal fashion. Based on this, they proposed that the accumulation of viroid RNA in the nucleus of these plants allowed some of the molecules to hybridize to the homologous sequences introduced into the chromosomes. The resulting triple-helical structure was then thought to be recognized by a DNA methylase whose function was to methylate any gene that produced more messenger than could be exported efficiently. In this case, the methylation only blocked expression of the cDNA copy of the gene while the viroids continued to replicate normally.

The viroid RNA is very different from any transcript known to occur in healthy plant cells in terms of its shape, untranslatability, and unusual abundance during later stages of infection. Nevertheless, its effect on an integrated copy of itself provides an example for silencing of more weakly expressed genes. English *et al.* (1996) have suggested that transgenes sometimes integrate near a DNA sequence that directly or indirectly influences 3′-end formation of the primary transcript. The resulting aberrant RNAs (aRNAs; English *et al.*, 1996), or perhaps any transcript recognized as having accumulated to excess (Lindbo *et al.*, 1993), would become substrates for a host-encoded RNA-dependent RNA polymerase (RdRP) that makes a complementary antisense RNA (asRNA). This asRNA could then bind back to its template triggering methylation as proposed by Wassenegger *et al.* (1994) or alternatively, be exported to the cytoplasm where it could bind to any homologous mRNA blocking its translation and/or marking both RNAs for degradation.

Our model shares some similarities with this, but differs in two details. Like Lindbo *et al.* (1993) and English *et al.* (1996), we believe that nuclear transcripts must begin correctly if they are to be made at all. Unlike them, we believe that transgenes (or translocated genes, genes near some deletions, or inversions) are transcribed normally. In our model, problems arise if the gene is not integrated near a nuclear processing tract (Carter *et al.*, 1993; Xing *et al.*, 1993). We believe that these pathways may be as intricate, as ordered, and as essential for mRNA export as secretion pathways are for protein export in bacteria. Without a nuclear processing tract to the outside, some of the nuclear RNA of the transgene might diffuse elsewhere and enter one of the hundreds or thousands of other export channels functioning at any single time, but most of the RNA would concentrate in the vicinity of its template. Genes without these tracts would appear to be transcribed normally, but little of the RNA would leave the nucleus to be translated. In some cases, this RNA could bind back to the gene to form a triple helix that could physically block further transcription until the complex dissociated or was cleared by a complex-specific RNAse. When this happened, genes would appear to be blocked transcriptionally even when there was no detectable change in methylation. The equilibrium between the free and bound RNA could determine the level of transcription of the affected template. This could, in some cases, lead to the intermediate levels of expression that we have generally termed position-effect variation. On the other hand, if the triple-helix persisted, there would be a chance that the promoter sequence could be methylated creating 'permanent' silencing.

Any gene could be affected by this mechanism, regardless of its level of expression. Nevertheless, genes expressed to higher levels should be more likely to saturate their nearest processing tracts and so be most likely to suppress themselves. If the nuclear RNA concentration decreased, transcription would begin again until the block was re-established. This hypothesis is fairly robust. For example, our model would work just as well if the introduced construct were expressing antisense RNA rather than sense RNA. While there is little doubt anti*sense* RNA *affects* homologous genes by forming an asRNA: sense RNA

complex, there are cases where mRNA levels have been reduced disproportionately to the levels of asRNA that have been found (van der Krol *et al.*, 1990b). These discrepancies might be explained if some plants failed to export all of the nuclear asRNA so that it could completely or partially interfere with transcription of itself and the gene of its intended target.

Our model also accounts for apparent threshold effects of gene expression on silencing, and begins to address where and when they have been observed. The rate of accumulation of nuclear RNAs should depend on both the rate of diffusion through the nucleus and on the general affinity of the nuclear RNA for its template. The affinity, in turn, should depend to a large extent on the RNA secondary structure and the G/C content of the template. We suggest that the deletion analysis English *et al.* (1996) performed may have identified the region of their gene making the most stable RNA–DNA hybrid. We also suggest that the correlation between haploidy and silencing, or the gradual increase in silencing during development (de Carvalho *et al.*, 1992), reflects differences in the number or organization of processing tracts in each phase of growth. Finally, we believe that many transcriptionally silent regions of the genome purposefully lack nuclear processing tracts. As a result, genes introduced nearby may have no connections to the channels needed for RNA processing and export. Heterochromatin and methylation may have more direct effects on template accessibility but triple-helix formation resulting from a failure to export would exasperate those problems.

As the excess primary transcript diffuses around the nucleus, some might bind to homologous sequences elsewhere leading to transcriptional co-suppression. Unlike Lindbo *et al.* (1993) and English *et al.* (1996), we do not believe that the accumulating RNAs have to be copied by RdRP into asRNA. While finding asRNA would not alter our model, it does seem inconsistent with those experiments that failed to find any asRNA or to correlate what was found with the level of co-suppression (Van Blokland *et al.*, 1994; de Carvalho Niebel *et al.*, 1995).

There are several points in common between the two models. Each, for example, assumes a DNA–RNA hybrid promotes gene methylation. Each also tacitly implies transgenes introduced into their homologous positions in the genome by recombination would not be susceptible to silencing. Finally, each of the two models has some predictable features that could be investigated (see below). However, neither appears to explain all the observations in the literature. For example, as presented so far, neither explains how a promoterless gene can silence a normal one (Van Blokland *et al.*, 1994). In a similar way, neither explains aspects of paramutation (Patterson *et al.*, 1993) or of transcriptional silencing as studied by Matzke *et al.* (1989; Park *et al.*, 1996). The rules governing these examples may indeed be unrelated to those for post-transcriptional silencing. On the other hand, one addition to our model might provide a means to account for even these cases.

We suggest that genes might synapse occasionally with homologous copies of their promoter or coding region located at ectopic sites. The propensity to synapse would depend in part on the size of the homologous region and the probability that random walk processes could bring the sequences on different chromosomes together during the period the genes were active. Tandem or inverted duplications

of the T-DNA at a single locus might be able to fold back on themselves depending on whether the torsion which that would generate could be relieved. Earlier models have also employed this feature, but these pairings were thought to suppress transcription by unidirectionally transmitting the chromatin structure of one allele to the other (Patterson *et al.*, 1993). Our model could account for post-transcriptional effects if the unusual synapsis disturbed the formation of nuclear processing tracts. In our model, post-transcriptionally co-suppressed genes are affected because they pair with a homologous gene in an inappropriate location. When two or more non-allelic copies are present in the nucleus, synapsis between regions of otherwise non-homologous chromosomes could distort nuclear structures disrupting the continuity of the processing tracts so that some primary transcripts would not be able to leave the nucleus. Nuclear run-off assays would detect little difference between the transcription of these genes and those that were not silenced.

In the simplest case, ectopic synapsing between two chromosomes might impair not only the genes directly aligned, but also the flanking but non-homologous genes as well. However, we hesitate to propose this is an inevitable consequence of the process because many factors can modulate the outcome. Factors that would determine whether nearby loci would be affected like the silenced ones are: (1) the location of the next-closest processing sites, (2) the distance between the affected locus and its neighbours and the amount of bending permited there, (3) the ability of topoisomerase to relieve some of the torsion between the synapsed and unsynapsed sequences, (4) the particular rates of accumulation of transcripts from adjacent genes, and (5) the sequence-dependent propensity of each transcript species to bind its template. Nevertheless, studies of changes in expression of genes adjacent to silenced genes will be important for both our model and model 1.

Testing the Hypotheses

If English *et al.* (1996) are correct, the 5′-end of the asRNA should be complementary to the 3′-end of the silenced gene. Altering the secondary structure or base composition of this region should always alter the degree of phenotypic suppression. On the other hand, if our model is correct, the efficiency of silencing should depend primarily on the length of homology and, in some cases, the affinity of the accumulating RNA for its target, regardless of its position in the gene. English *et al.* (1996) also state plants should have an RdRP capable of (a) recognizing unusual 3′-ends and (b) synthesizing complementary copies from a diverse array of templates. aRNAs presumably differ in their termination point from normal nuclear RNAs: if these aRNAs could be processed correctly and polyadenylated, they would be functionally indistinguishable from normal RNAs. Yet if they lack this tail, it is unclear what common sequence at the 3′-end could be used to prime the new strand so that the asRNA could be made.

If our model proves to be correct, *in-situ* studies of RNA export (as done by Carter *et al.*, 1993 and Xing *et al.*, 1993), should show transcripts accumulating around transgenes prone to silencing but not those transgenes showing consistent

expression. Genes showing consistent expression should have a way of passing the primary transcript along to a nearby processing tract. Based on this, we presume transgenes showing consistent expression have integrated near a locus anchored to a processing tract. It is possible these tracts assemble wherever there are patches of open chromatin. If so, LCRs might directly or indirectly be involved in establishing the beginning of the processing tract which accepts the RNA after termination. Incorporating such sites in a transgene should prevent any such localized RNA accumulation.

Both models explain gene-specific methylation by means of RNA–DNA hybrids and a methyltransferase capable of recognizing them. One might therefore see less transcriptional silencing in *Arabidopsis thaliana* mutants defective in a protein regulating methyltransferase : DNA target interactions (Kakutani *et al.*, 1995). Adding additional methyltransferases might alter the frequency of silencing at different developmental stages as seen in *Drosophila* (Wines *et al.*, 1996). English *et al.* suggested methylation might promote aRNA synthesis, but did not make this a condition of their hypothesis. In our model, post-transcriptional silencing should not be affected. Additional insights will no doubt come from studies of mutants with reduced frequencies of silencing which could be altering processing tract formation, triple-helix formation, RNA-dependent DNA silencing, or a process that has not yet been identified (Dehio and Schell, 1994).

Genetic Engineering of Plants for Disease Resistance

There is one additional set of experiments that needs to be addressed to accommodate it with the two models. This set consists of several recent studies that have shown that in some cases virus resistance in transgenic plants expressing viral genome sequences is correlated with post-transcriptional gene silencing. Lindbo *et al.* (1993) reported that transgenic plants expressing either a full-length or a truncated form of tobacco etch virus (TEV) coat protein were initially susceptible to TEV (an RNA virus) infection. Then, 3–5 weeks after inoculation, new stem and leaf tissues arose from these transformants that appeared symptomless, virus-free, and more or less 'recovered' from infection. Challenge experiments revealed that the recovered transgenic tissue was susceptible to potato virus Y, a closely related virus, but was resistant to re-infection with TEV. Nuclear run-off assays showed that although transgene transcription proceeded at comparable rates in both uninoculated and recovered transgenic plants, the cytoplasmic accumulation of the transgene mRNA was 12–22-fold less in recovered tissues than in uninoculated transformant tissue of the same developmental stage. These results as well as others have led researchers to speculate that RNA-mediated virus resistance is a form of post-transcriptional silencing caused when a specific threshold for a given messenger in the cytoplasm is exceeded. RNA accumulating beyond this level is marked by either a protein or an RNA for cytoplasmic degradation (Lindbo *et al.*, 1993; Smith *et al.*, 1994; Mueller *et al.*, 1995; English *et al.*, 1996). English *et al.* pointed out, though, that there has not been a consistent correlation

between silencing and the magnitude of expression. This in turn led to their suggestion that the cell recognized qualitative differences such as aRNA formation rather than RNA abundance. In addition, they proposed that either aRNA, or asRNA made from it by endogenous RdRP, is able to enter the nucleus and trigger further suppression through transgene methylation. The question that was not answered here is what structural feature is being used by RdRP or any other molecule to distinguish RNA from aRNA, and what type of primer used by RdRP is able to pair with so many different aberrant templates.

We suggest that a variation on our model might account for some cases where virus resistance correlates with gene silencing, but this will require a reevaluation of some experiments on potyviral replication. Potyviruses are positive-sense RNA viruses encoding a large polyprotein precursor that is proteolytically processed into eight or more mature proteins. It is assumed that these viruses only replicate in the cytoplasm since virus particles accumulate there. However, other properties of the virus could be consistent with nuclear replication. For example, the viral RNA genome is polyadenylated. Nuclear-encoded genes use this tail not only for RNA stabilization but also for nuclear export (Huang and Carmichael, 1996). In addition, the virus makes two replication-associated inclusion proteins, Nla and Nlb, that are localized predominantly in the nucleus (Knuhtsen *et al.*, 1974). Restrepo *et al.* (1990) showed that fusion of either Nla or Nlb protein to the β-glucuronidase (GUS) reporter enzyme targets the reporter to the nucleus. This localization could be rationalized if one stage of potyvirus replication takes place in the nucleus where there are high concentrations of all the necessary components for RNA synthesis such as nucleotides and RNA processing enzymes. The RNA could then be exported to the cytoplasm like any other RNA, be translated, and finally assembled into viral particles.

According to our model, the nuclear copies of the viral genome either accumulate quite rapidly, or are processed and exported somewhat slowly because they are not linked directly to nuclear processing tracts. Neither property would affect viral proliferation in untransformed plants, but plants with an integrated copy of a viral gene might then accumulate partially complementary mRNA–genome RNA and RNA–DNA hybrid molecules. Formation of the latter could block mRNA synthesis by processes described earlier. Formation of the former would generate molecules unlike anything found during normal viral infection. This aberrant structure could sterically block further replication like the RNA–DNA complex blocks transcription. The hybrid might also be exported less efficiently than single-strand RNA. Any duplex that is exported intact or that forms afterwards in the cytoplasm could be recognized as unusual and degraded by RNAses such as those found in animals (Zhou *et al.*, 1993).

Summary

Despite the efforts of a number of laboratories there is still no single, persuasive explanation for the variability in expression that has been seen in some transgenic

plants. Phenotypic variability and gene silencing appear to be stimulated by *in-vitro* culture conditions, and depend at least in part on the sequence of the promoter and coding region. Homology between parts of the transgene and other genes in the genome may cause the random inactivation of the transgene or co-suppression of the endogenous homologue. Silencing can change over the course of development of the plant after germination, and can be altered by undefined environmental factors. Many cases of silencing are reversible and likely to be epigenetic. While none of the genes examined have been mutated, it is still possible that future examples of silencing will prove to be due to changes in the DNA sequence.

Most recent reviews have distinguished two different forms of silencing: transcriptional and post-transcriptional. Transcriptional silencing appears to be expression-independent. It is presumed to be correlated with the proximity of the transgene to normally silent regions of a chromosome rather than to how much RNA is being made. Processes like methylation may extend from these untranscriable regions to the site where transgenes have integrated. As a result of position-dependent methylation or heterochromatinization, these transgenes would not be transcribed. This is contrasted with post-transcriptional silencing where nuclear RNA is produced at expected levels but simply not exported, or is transported but quickly degraded in the cytoplasm. This form of silencing is dependent on both the position (since the same construct can behave normally in other transformed individuals where it lies in a different chromosomal location) and the expression of the transgene.

Though there may be several causes for gene silencing, we have attempted to construct a single model capable of explaining the majority of the observations. We believe that all silenced genes can be transcribed normally but if the genes are not near RNA processing tracts, they do not get exported efficiently. As a consequence, they tend to accumulate around their template and are not exported. The transcripts can also inhibit further transcription through the formation of RNA–DNA triple-helices. If these transcripts persist there, the templates tend to be silenced permanently through methylation of the gene by enzymes acting on triple-helical substrates. Genes in different parts of the genome may occasionally synapse, but in so doing, they would distort the nucleoplasm and distort some of their nearby RNA processing tracts. As a result, these messengers also would accumulate and silence their genes. This hypothesis could be tested using methods already developed for studies on mammalian genomes.

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28. Transposable Elements and Genetic Variation

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1. Introduction

Fifty years ago the first transposable elements were discovered in *Zea mays* through the pioneering work of Barbara McClintock. Meanwhile transposons were found in all kinds of living organisms and several of these elements have been investigated at the molecular level.

From a classical geneticist's or breeder's point of view, chromosomes and genomes seem to have highly static arrangements of genes and polynucleotides. Genes or gene loci are arranged on the chromosomes like pearls on a string and never change their position. Based on this genome stability, physical maps can be constructed and the position of any single locus within a genome can be defined. In contrast, from a molecular geneticist's point of view, the DNA structure is not rigid at all. Many recombination, rearrangement and mutation events cause structural changes and even instability within the genome. These mutational changes and DNA rearrangements are partially caused by genome-inherent mobile DNA sequences, such as plasmids (in bacteria and organelles), virions or transposons. Almost all mobile DNA sequences share some common features. Thus the question arises whether these different types of vagabonds in different genomes arose from the same origin or by several independent evolutionary events.

Transposable elements – also called 'jumping genes' – are discrete DNA sequences enable to transport directly to other locations within a genome. These changes in positions are normally relatively rare events. Sometimes the frequency of transposition can increase dramatically, especially when mutagens or stress factors are acting in the environment. In contrast to point mutations, the activity of transposons affects larger regions within the host genome. The mobilization of transposable elements leads to a variety of mutations including insertions, deletions, duplications, inversions, translocations and rearrangements in genomic sequences as well as multiplex changes of chromosomal structures. Transposons are able to modulate the activity of genes or to switch gene functions from an active into an inactive state or vice-versa. They can also alter the function of genes by simply placing them in new regulatory situations. Furthermore, several types of genome rearrangements caused by recombination or multiplication of transposable elements are thought to be important in evolution (Berg and Howe, 1989).

So far, transposable elements have been detected in all organisms, when examined in detail with respect to the presence of these mobile DNA elements. They can occupy between 5% and 20% of the genome (Finnegan and Fawcett, 1986;

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Kochanek *et al.*, 1993; Suoniemi *et al.*, 1996). Transposons were first identified in *Zea mays*, described by the pioneering work of Barbara McClintock (1948). McClintock originally used the term 'controlling elements' because of their obvious influence on gene expression. Insertion mutations caused by transposable elements reduce or prevent gene expression. In many cases, the restoration of the wild type occurs after excision of the element. For a long time the functional mechanism of these jumping genes remained unexplained. Almost 40 years after their discovery the first element, called *Activator*, was successfully isolated from the maize genome (Fedoroff *et al.*, 1983; Behrens *et al.*, 1984) and characterized at the molecular level (Pohlmann *et al.*, 1984; Müller-Neumann *et al.*, 1984). Twenty years after the discovery of the existence of transposable elements in maize, the same phenomenon was described in bacteria (Saedler and Starlinger, 1967; Jordan *et al.*, 1968; Shapiro, 1969). It was found that antibiotic-resistance bacterial genes were often encoded by mobile DNA elements. These resistance genes changed their position within the bacterial genome and can be transferred to other bacteria of the same species or even a different species.

Similarly, within the past 20 years, many different transposable elements have been discovered and characterized in fungi and animals, for example in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and in human genomes. It is obvious today that transposable elements occur in all kinds of living organisms and play an important role in those processes responsible for evolution of genomes. The work done on mobile elements within recent decades has shed light on the phenomenon of genome variability.

2. Types of Transposable Elements

Three groups of DNA transposons were defined on the basis of extensive investigations done on bacteria in the 1970s and 1980s. These groups are: (i) insertion sequences (*IS*), (ii) transposons and (iii) transposable bacteriophages.

2.1. Insertional Sequences (IS)

The simplest transposons were called insertion sequences. They seem to occur in all genomes, and the best characterized ones come from bacteria. Each *IS* is different in sequence from other elements but they all share some common structural features. They are DNA molecules of a distinct length, usually between 800 and 1500 bp (or even shorter, when non-autonomous), flanked by inverted terminal repeats and generally produce short repeats in the target DNA during insertion. The most common length for the direct repeats produced in the target locus is 8–9 bp. Up to now, more than ten different *IS* (Birkenbihl and Vielmetter, 1989) have been identified as a single or multiple copies in *Escherichia coli* genomes. *IS* code for a transposase protein only. The non-random insertion of *IS* can only be monitored by their ability to change the gene expression. Gene functions may be affected by insertion mutagenesis itself or by promoter and terminator properties of

the *IS*. This could be the reason for: (a) overexpression of genes (Saedler *et al.*, 1980); (b) blockage of gene expression (Blasband *et al.*, 1986); and (c) gene loci that show otherwise variable expression (Reynolds *et al.*, 1981). Besides altering the gene expression, *IS* can also result in internal genome rearrangements or deletion of genome regions.

2.2. Transposons

Transposons are widespread in bacteria, animals and plants. They have inverted repeats at their termini and generate short direct duplications of the target DNA sequences during integration, which are known as footprints. Bacterial transposons or Tn-elements (Kleckner, 1981; Sherratt, 1989) are more complex than *IS* and have a length about 5 kbp or even larger. They contain two copies of an *IS* flanked by inverted repeats. These two *IS* are able to transpose themselves and also any sequence between them. A common feature among compound transposons in *Escherichia coli* is that only one of the *IS* is active. This mechanism guarantees the favourable transposition of the whole Tn instead of the single *IS*. In bacterial transposons the central region, apart from encoding the translocation machinery, usually codes for additional genes such as antibiotic resistances (Kleckner, 1989; Fig. 1). In plants, transposons often have a simple structure and harbour a single gene such as the *Ac* element (Fig. 1) of *Zea mays* or a few genes, as with *Spm* or *Tam1* (see also Fig. 1). These genes are responsible for the translocation of the elements themselves (the transposase gene) or the gene products are modulating factors during the transposition event (e.g. enhancers, modulators or suppressors). In plant genomes, DNA-based transposable elements normally are present in a few to many copies. These copies are either similar to each other or very different. In the latter case, they develop transposon families. The members of each family can be classified either as autonomous or non-autonomous elements. The autonomous elements are characterized by a continuous activity of translocation in the genome. Non-autonomous transposons are thought to be partially derived from autonomous elements by the loss of transposase gene function which is essential for the translocation. This is the case, for instance, for the elements of the *Ac/Ds* family of transposons in *Zea mays* (Table 1) where it is evident that many naturally occuring *Ds* elements arose from *Ac* by either deletions or other mutations. They can be activated *in trans* by the presence of an active autonomous element of the same family.

2.3. Transposable Bacteriophages

The third class of transposons, the transposable virions (e.g. mutator-bacteriophage *Mu*) infect a sensitive host by injecting their mature viral DNA and associated proteins into the cell. This integration process of non-replicative transposition leads to mutation by destroying chromosome loci in the donor molecule. The copy number in the genome can be amplified about 100-fold by replicative transposition, requiring certain *Mu* proteins and the host replication system. The

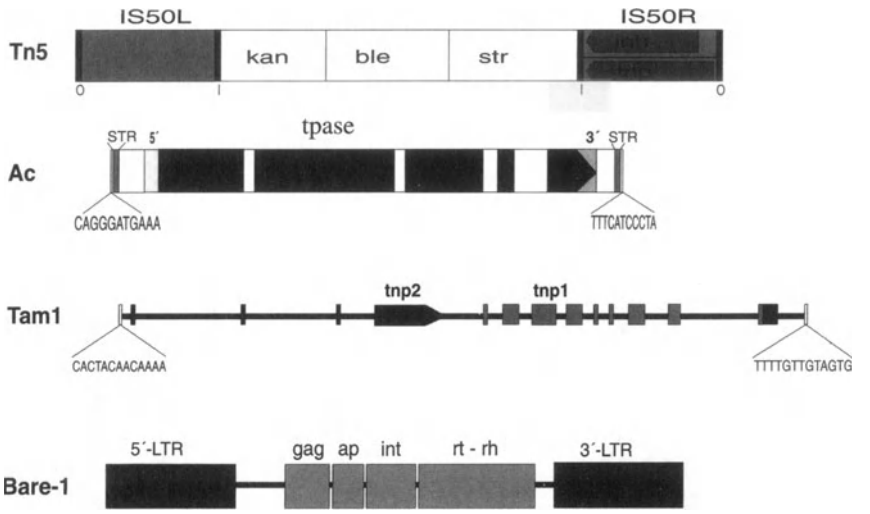

Figure 1. Transposable element structures. From top to bottom: Bacterial transposon ***Tn5*** (Berg and Howe, 1989), representing the class of bacterial composite transposons. It consists of two terminal insertion sequences (*IS50R*, *IS50L*) with terminal inverted repeats (I, O). The *IS50R* codes for two proteins, the transposase (*tnp*) and a smaller inhibitor (*inh*). The central part is covered by three antibiotic resistance genes coding for aminoglycoside phosphotransferase (*kan*), bleomycin resistance protein (*ble*) and streptomycin phosphotransferase (*str*). Autonomous transposable element ***Activator (Ac)*** from *Zea mays* (Fedoroff, 1989). A single mosaic gene (five exons, four introns) codes for the transposase (*tpase*) and is flanked by subterminal regions (*STR*) where conserved sequence motifs are located that are necessary for initial binding of the transposase. The outer sequences are the imperfect terminal inverted repeats of 11 bp. The autonomous transposable element ***Tam1*** (Nacken *et al.*, 1991; Weil and Wessler, 1990) from *Antirrhinum majus* codes for two proteins, a putative DNA binding protein (*tnp1*), the transposase (*tnp2*) and is flanked by 13 bp perfect TIR. The retrotransposon ***Bare1*** (Suoniemi *et al.*, 1996) is flanked by 3′ and 5′ long terminal repeats. In the centre conserved sequences are located for a Gag-protein domain (*gag*), an aspartic proteinase (*ap*), a polymerase containing integrase (*int*), for revertase (*rt*) and endonuclease H (*rh*) domains

product of replicative transposition is a cointegrate between transposon and host DNA. It is not clear whether replicative transposition, also described for some plant transposons, always occurs by excision of the elements (Walbot and Warren, 1988). In contrast to all other classes of transposable elements, so far transposable DNA viruses have not been found in plants.

2.4. Retrotransposons

Besides DNA-based transposons, there exists another group of transposable elements known as retroelements, which are the most widespread class of eukaryotic transposable elements. They include retroviruses as well as long terminal repeat (LTR) and non-LTR retrotransposons. These elements organize their own transposition and multiplication via RNA intermediates (Boeke *et al.*, 1985) and share several similarities to retroviruses. Active retrotransposons, like retroviruses,

Table 1. Selection of transposable elements in important plant species

Plant species	Transposable element	Reference
Antirrhinum majus	*Tam1*	Bonas *et al.*, 1984
	Tam2	Upadhyaya *et al.*, 1985
	Tam3	Sommer *et al.*, 1985; Hehl *et al.*, 1991
	Tam7, Tam9	Schwarz-Sommer *et al.*, 1990
Arabidopsis thaliana	*Ta1*	Voytas and Ausubel, 1988
	Ta1, Ta2, Ta3	Konienczny *et al.*, 1990
	Tag1	Tsay *et al.*, 1993
	Tat1	Peleman *et al.*, 1991
Nicotiana alata	*Tna1*	Royo *et al.*, 1996
Nicotiana plumbaginifolia	Tnp1	Meyer *et al.*, 1994
Nicotiana tabacum	*Tnt1*	Grandbastien *et al.*, 1989
	Tto1	Hirochika, 1993
	Npg1	Tebbutt and Londsdle, 1993
Petunia hybrida	*Act1*	Huits *et al.*, 1995
	dTph1	Gerats *et al.*, 1990
	dTph1–3; dTph4	Renckens *et al.*, 1996
Zea mays	*Ac/Ds*	Fedoroff *et al.*, 1983
	Bg/rbg	Salamini, 1980; Motto *et al.*, 1989
	Bs17	Mottinger *et al.*, 1984
	B5	Varagona *et al.*, 1992
	Cin1	Shepherd *et al.*, 1984
	Cin4	Schwarz-Sommer *et al.*, 1987
	Cy	Schnable and Peterson, 1986
	Dt	Dörshug, 1968
	En/Spm	Peterson, 1953; Pereira *et al.*, 1986; Masson *et al.*, 1991
	Mu, Mu1	Robertson, 1978
	retro	Wessler and Varagona, 1985
	rDt	Brown *et al.*, 1989a
	Stonor	Varagona *et al.*, 1992
	Uq	Friedemann and Peterson, 1982; Pisabaro *et al.* 1991
	Tourist-Zm1	Bureau and Wessler, 1992

contain a gene for a RNA-dependent reverse transcriptase. This gene product enables them to transcribe the RNA intermediates into DNA and thus to manifest in cells by integration of DNA copies into the host genome (Grandbastien, 1992). Copies of retrotransposons integrate as stable elements in the host genome but do not possess the ability to excise. They can be dispersed all over the genome via reverse transcription of their RNA intermediates, and thereby an increase of retrotransposons in the cells can be monitored.

Well-described retrotransposons include the various *Ty* elements of *Saccharomyces cerevisiae* (Stucka *et al.*, 1992). Evidence was given for the different elements of this group, that they can derive either from each other or from independent ancestors. Retrotransposons were also found in *Drosophila*

melanogaster and Drosophilidae, for instance *copia* and *copia*-like sequences respectively, but *copia*- as well as *Ty*-like elements also occur in plants (Flavell, 1992; Pearce *et al.*, 1996; Suoniemi *et al.*, 1996). They are about 5–9 kbp in size and their termini consist of direct duplications of 200–500 bp, being referred to as long terminal repeats (Bingham and Zachar, 1989). One extensively investigated example of a plant (LTR) retrotransposon is *Bare-1* from *Hordeum vulgare* (Fig. 1; Suoniemi *et al.*, 1996).

In humans and animals, short middle-repetitive sequences can be found, that are randomly dispersed all over the genome and are called *Alu* sequences (in humans) or *Alu*-like sequences (in other mammals). These sequences, that are thought to be of retrotransposon origin, seem to have an important function in genome rearrangements and may serve as replication initiators.

3. Transposable Elements and their Roles in Plants

Transposons are present in many plant species, although jumping genes have been investigated and characterized in detail in only a few species. Transposable elements are mostly known in the plant species *Zea mays*, *Antirrhinum majus*, *Arabidopsis thaliana*, *Nicotiana* species and *Petunia hybrida* (Table 1). The best characterized transposable elements include different transposon families in *Zea mays* (Fedoroff *et al.*, 1983; Hartings *et al.*, 1991; Masson *et al.*, 1991), *Tam* elements in *Antirrhinum majus* (Bonas *et al.*, 1984; Upadhyaya *et al.*, 1985; Hehl *et al.*, 1987, 1991; Schwarz -Sommer *et al.*, 1990) and the retrotransposons *Tnt1* (Grandbastien *et al.*, 1989), *Tto1* (Hirochika and Hirochika, 1993) from *Nicotiana tabacum* as well as the *Hordeum vulgare* retrotransposon *Bare-1* (Manninen and Schulman, 1993; Suoniemi *et al.*, 1996; see Fig. 1). Retroelements are by far the largest and most widespread class of mobile genetic elements. They were found to be ubiquitous in plants (Grandbastien, 1992; Voytas *et al.*, 1992).

3.1. Maize Transposable Elements

Up to now, at least nine genetically distinguishable regulator-receptor transposable element systems have been detected in maize (Pan and Peterson, 1991) as well as several other transposons, and among them also different retrotransposons (see Tables 1 and 2). Three maize element families have been studied very extensively at the molecular level. These are the *Activator/Dissociation* (*Ac/Ds*), the *Suppressor–Mutator* (Enhancer/Inhibitor, *En/Spm*) and the *Mutator* elements (*Mu*).

3.1.1. Activator/Dissociation elements

The best characterized plant transposable elements are the members of the *Ac/Ds* family, first described by McClintock (1948, 1956, 1967). Initially, mutations having arisen from the action of these transposons were divided into two different

Table 2. Maize loci tagged by transposable elements

Locus	Name	Status	Transposon	Reference
a 1	anthocyanin less 1	ps	*dSpm*	Masson *et al.*, 1987
			Dt/rDt	Brown *et al.*, 1989a
			En	Peterson, 1985
a 2	anthocyanin less 2	t	*cy/rcy: Mu 7*	Menssen *et al.* 1990
			dSpm	Mensen *et al.*, 1990
			En	Peterson, 1978
			Mu 1	Walbot, 1992b
adh 1	alcohol dehydrogenase 1	s	*BS 1*	Johns *et al.*, 1989
			Ds	Sutton *et al.*, 1984
			Mu	Taylor and Walbot, 1985; Freeling *et al.*, 1982
ae 1	amylose extender 1	t	*Mu 1*	Stinard and Robertson, 1988
an 1	anther ear 1	s	*Mu 2*	Bensen *et al.*, 1995
b 1	coloured plant 1	s	*Ds*	Clark *et al.*, 1990
bt 1	brittle endosperm 1	s	*dSpm*	Sullivan *et al.*, 1991
			Mu	Neuffer *et al.*, 1996
bz 1	bronze 1	s	*Ac*	Fedoroff *et al.*, 1984; Ralston *et al.*, 1989
			Ds	McClintock, 1956, 1962; Scheifelbein *et al.*, 1988
			dSpm	Nelson and Klein, 1984
			Mu 1	Brown *et al.*, 1989b
bz 2	bronze 2	s	*Ds*	Chandlee, 1990
			Mu	Nash *et al.*, 1990
			Mu1 Mu9	Walbot, 1992b
c 1	coloured aleurone 1	s	*Ds*	Scheffler *et al.*, 1994
			dSpm	Cone *et al.*, 1986
			En	Reddy and Peterson, 1983
c 2	colourless 2	s	*En/Spm*	McClintock, 1967
			En	Chang and Peterson, 1994
			Mu 1	Walbot, 1992b
			MpI	Weydemann *et al.*, 1988
cps 1	chloroplast protein synthesis 1	t	*Mu*	Barkan, 1993
cps 2	chloroplast protein synthesis 2	t	*Mu*	Barkan, 1993
d 3	dwarf plant 3	s	*Mu 8*	Neuffer *et al.*, 1996
g 2	golden plant 2	t	*dSpm*	Peterson, 1995
gl 1	glossy 1	t	*Mu 1*	Neuffer *et al.*, 1996
gl 8	glossy 8	s	*Mu8*	Schnable and Wise., 1994
gl 15	glossy 15	t	*dSpm*	Moose and Sisco, 1994
hcf 106	high chlorophyll fluorescence	t	*Mu 1*	Das and Martienssen, 1995
hm 1	*helminthosporium carbonum* susceptibility 1	s	*dHbr*	Meeley *et al.*, 1992
			Mu 1	Meeley *et al.*, 1992
			Mu 3	Meeley *et al.*, 1992
			dSpm	Meeley *et al.*, 1992
id	indeterminate growth 1	t	*Ds*	Colasanti and Sundaresan, 1995
ij 1	iojap striping 1	s	*Ds*	Horsington and Coe, 1990
			Mu 1	Han *et al.*, 1992

Table 2. Maize loci tagged by transposable elements (contd)

Locus	Name	Status	Transposon	Reference
les-J2552*	lesion*-J2552	t	*Mu 1*	Johal *et al.* 1994
kn 1	knotted 1	t	*Ds*	Hake *et al.*, 1989
			Mu 1	Veil *et al.*, 1991
lg 2	ligule-less 2	t	*Mu 8*	Walsh and Freeling, 1995
lg 3	ligule-less 3	s	*Mu*	Neuffer *et al.*, 1996
lls 1	lethal leaf spot 1	t	*Mu 1*	Johal *et al.*, 1994
ms 45	male sterile 45	t	*Ac*	Chasan, 1994
o 2	opaque endosperm 2	s	*Ac*	Michel *et al.*, 1995
			Bg	Michel *et al.*, 1995
			Ds	Motto *et al.*, 1986
			En/Spm	Michel *et al.*, 1995
p 1	pericarp colour 1	s	*Ac*	Greenblatt, 1984
pr 1	red aleurone 1	t	Ac	Scanlon *et al.*, 1994
ps 1	pink scutellum 1	t	*Ac*	Robertson, 1995
py1-tan	pigmy plant 1	t	*Mu*	Neuffer *et al.*, 1996
r 1	coloured 1	t	*Ds*	Alleman and Kermicle, 1993
ren 2	reduced endosperm	t	*Mu*	Scalon *et al.*, 1994
ren 3	reduced endosperm 3	t	*Mu*	Scanlon et., 1994
rf 2	restorer of fertility 2	s	*Mu*	Schnable and Wise, 1994
			Spm/En	Schnable and Wise, 1994
rs 1	rough sheath 1	s	*Mu 6/7*	Becraft and Freeling, 1991
sh 1	shrunken 1	s	*Ds*	Courage-Tebbe *et al.* 1983
				Döring and Starlinger, 1984
			Tourist A	Zack *et al.*, 1986
sh 2	shrunken 2	s	*Ds*	Giroux *et al.*, 1993
			Ils 1	Alrefai *et al.*, 1994
su 1	sugary 1	s	*Mu 1*	James and Myers, 1994
te 1	terminal ear 1	t	*Mu 8*	Neuffer *et al.*, 1996
ts	tassel seed 2	s	*Ac*	De Long *et al.*, 1993
vp 1	viviparous 1	s	*Mu*	McCarty *et al.*, 1989
wx 1	waxy 1	s	*Ac*	McClintock, 1963
				Fedoroff *et al.*, 1983
				Behrens *et al.*, 1984
			Bg	Hartings, 1990
			Ds	Varagona and Wessler, 1990
				Weil *et al.*, 1992
			En/Spm	Pereira *et al.*, 1986
			dSon	McClintock, 1961
			Mu 8	Gierl *et al.*, 1985
			magellan	Fleenor *et al.*, 1990
			Retro trans	Purugganan and Wessler, 1994
			Hopscotch	Varagona *et al.*, 1992
			Tourist A	Wessler and Varagona, 1985
			Stonor	Wessler and Varagona, 1985
y 1	white 1	s	*Mu*	Beckner *et al.*, 1990

s: Sequenced; ps: partially sequenced; t: locus tagged.

types of insertion mutations. One group is inherently unstable and the other is unstable in the presence of a complete transposable element in the genome only. These two types of mutations were defined to be caused by autonomous *Ac* and non-autonomous *Ds* elements. Non-autonomous elements, like *Ds* and *dSpm* in the case of the *En/Spm* family, have high copy number (30–60) in the genome. The autonomous elements, like *Ac* and *Spm*, however, have low copy number (1–9) (O'Reilly *et al.*, 1985; Lewin, 1991). *Ds* elements always require the *Activator* to transpose. They can integrate into genes resulting in a null phenotype as well as in decreases of gene expression, or they can also initiate chromosome breakage. McClintock (1948) was the first to describe the ability of a *Ds* element to transpose, and that revealed as a mutation at the *c* locus on the short arm of chromosome 9 of maize. As long as *Ac* elements remain inactive, the insertion mutations caused by *Ac* are comparable with *Ds* effects. However, they differ from true *Ds* elements by their ability to return to active forms and thus to continue transposition. The *Ac* element was isolated from a *waxy* locus (Fedoroff *et al.*, 1983; Behrens *et al.*, 1984; Pohlmann *et al.*, 1984; Müller-Neumann *et al.*, 1984). The *Ac* element is defined by a length of 4.6 kbp and has 11 bp inverted imperfect terminal repeats (Fig. 1). The element possesses a single transcription unit that encodes a transposase which consists of 807 amino acids (Kunze *et al.*, 1987). In contrast to *Ac*, the group of *Ds* elements is heterologous in structure. On the one hand there are *Ds* elements that originate from *Ac* by deletion (e.g. *DS9*, *DS2d1*, *DS2d2* and *DS6*), whilst other *Ds* elements do not show any structural relation to *Ac* despite the terminal inverted repeats (*DS1* elements) (Sutton *et al.*, 1984; Peacock *et al.*, 1984). Their length varies from only a few hundred basepairs to almost the full length of *Ac*.

3.1.2. *Suppressor–Mutator* Elements

The *Suppressor–Mutator* element family can be divided into two main groups. In this family, the autonomous elements were termed as *Enhancer (En)* or *Spm* and the non-autonomous as *Inhibitor (I)* (Peterson, 1953) or defective *Spm* (*dSpm*) (Banks *et al.*, 1988). The autonomous *Spm* element is a member of the CACTA family of transposons having short (13 bp) terminal inverted repeats. *Spm* and *En* (cloned from the *waxy* locus) differ by four nucleotides in length and by sequence at single nucleotides only. The 8.3 kbp *Spm* element encodes a single transcript containing two open reading frames. Two proteins are encoded by this element. The protein *TNPA* binds to a 12 bp repeated sequence motif present in the subtermini of *Spm*. In this way, both ends of the element can come together in a zipper-like fashion (Frey *et al.*, 1990). This structure interacts with the second protein called *TNPD*, a proposed endonuclease, in order to catalyse the excision (Muszynski *et al.*, 1993). The mutator function of *Spm* has been described for cases where the wild type gene activity is restored after excision from mutable alleles. This results in a spotted phenotype which is due to the interaction of *TNPA* and *TNPD* with the elements to promote the excision rate. Besides the mutator function of *Spm*, these elements are mainly characterized by their ability

to modulate gene expression. Active *Spm* elements seem to be present in a few maize lines only (Peterson and Salamini, 1986). All of the *dSpm* show a closely related structure to their autonomous counterpart and differ from active *Spm* by some internal deletions (Menssen *et al.*, 1990).

3.1.3. Mutator Elements

The *Mutator (Mu)* element system in maize was originally characterized by the induction of very high frequency rates of mutations at a wide range of loci. These elements were able to increase the spontaneous mutation rate about 50-fold or more (Robertson, 1978). It was shown that *Mu* plants contained multiple copies (10 to more than 100 elements) in their genome. Six different classes of *Mu* transposable elements have been identified based on the similarity with their internal sequences (Hardeman and Chandler, 1993). They contain a variety of unique internal regions representing both evolutionally related and independently derived sequences. Most *Mu* containing maize lines harbour several different classes of *Mu* transposons, each of which can be present in high copies. In the *Mu* family, there are several elements with similar stuctural features, possessing long terminal inverted repeats and ranging in size from 1.4 to more than 5 kbp. However, smaller *Mu* elements of 1.1 and 0.8 kbp have also been cloned (Taylor *et al.*, 1986). Within this family, the subfamily *Mu1* is responsible for most of the *Mutator*-induced mutations. The *Mu1* element is 1.4 kbp in length and has 200 bp inverted repeats. It produces a 9 bp target duplication (Barker *et al.*, 1984). *Mu1* and *Mu1.7* elements are similar in sequence, with the exception that *Mu1.7* carries an additional 385 bp internal sequence. To date, no analogous features to members of the *Ac* or *Spm* elements have been found. Cresse *et al.* (1995) reported that *Mu1*-like elements preferentially insert in loci of the maize genome that are present in unique or low copy number. Walbot and Warren (1988) and Doseff *et al.* (1991) found that the excision of *Mu1* elements occurs late in somatic tissues and very rarely in the germline. For a long time the function of the *Mu* system remained tentative, because there was no identification of the element or gene responsible for *trans*-activating transposition. Chomet *et al.* (1991) showed that active *MuR1* can regulate the transposition of other *Mu1* elements. A similar element called *MuA* was identified by Qin and Ellingboe (1990). *MuA* is associated with the transposition of other *Mutator* elements both qualitatively and quantitatively.

Besides these three families, other transposable elements have been characterized in maize through cloning of insertions or at the molecular level. An updated list of maize transposable elements and the respective references are presented in Table 1 and Table 2. It has been shown that one maize line may contain elements of different transposable element systems. Schnable and Peterson (1986) found at least four different elements (*Cy, En, Ac, Dt*) in the so-called transposable element-laden (TEL) stock. Michel *et al.* (1995) isolated unstable alleles of the *opaque2* (*o2*) locus, assumed to be caused by an *Ac* insertion. Unexpectedly, they

found mutations due to elements of the *Spm* and *Bg* families. To date, however, there is no evidence for an interaction between different families of transposable elements (Wienand and Saedler, 1987).

3.2. Transposable Elements in Antirrhinum majus

Five different transposable elements have been characterized in more detail from *Antirrhinum majus* (snapdragon) due to their occurrence in specific loci responsible for flower pigmentation or for flower development (Table 1). *Tam1* (Fig. 1) is a transposable element isolated from the promoter region of an unstable *nivea* (chalcone synthase gene) locus (*niv rec-53*). *Tam1* is 15 kbp long and includes at least two genes that are transcribed to produce a 2.4 kb (*tnp1*) and a 5 kb (*tnp2*) mRNA. The 5 kb transcript contains an open reading frame, that shares 45% homology with the *tnpD* gene of *Spm* elements from maize (Nacken *et al.*, 1991). *Tam1* can be present in the genome between 15 and 20 copies. *Tam2* is 5 kbp in length and was localized in a further *niv* allele (*niv44*). It was isolated from the first intron of the *chs* gene. It has perfect terminal inverted repeats of 14 bp (Upadhyaya *et al.*, 1985). Although *Tam2* possesses all features of an autonomous transposable element, there was no indication of excision during plant development for a long time. Flowers of *niv44* allele plants show a stable white colour and at the molecular level no transposition could be detected. This could, however, be due to a very rare excision frequency which is far below the sensitivity of an experimental protocol. Both *Tam1* and *Tam2* generate a 3 bp target duplication. The 13 bp terminal inverted repeats of *Tam1* are identical to the first 13 bp of the terminal inverted repeats of *Tam2*. The first 200 bp of the left-hand terminus was also homologous to *Tam2*, thus indicating that these two transposons might have originated from the same ancestor. To prove this, Hehl *et al.* (1987) crossed the stable *niv44* mutation carrying *Tam2* and two stable *niv* lines (*niv46* and *niv49*) harbouring *Tam1* element derivatives. For the first time, the excision of *Tam2* elements could be shown.

Tam3 was inserted in the two unlinked loci, *nivea* and *pallida* (*pal*, codes for dihydro-flavonol 4-reductase). It was isolated from the *pal* promoter as well as from the *niv rec98* allele, where it is located 29 bp upstream of the TATA box. Both isolates share a length of 3.5 kbp and have 12 bp inverted repeats. They differ in their length of target duplication. In the *niv* allele *Tam3* generates an 8 bp duplication, whereas in the *pal* allele only a 5 bp duplication was reported (Martin *et al.*, 1985). The *Tam3* elements are present in 25–30 copies in the genome. *Tam3* can generate numerous alleles conferring a range of phenotypes at both the *pal* and *niv* loci due to high frequency of excision. The excision frequency of *Tam3* depends on the temperature and a repressor-like element called *Stabiliser* (Martin *et al.*, 1985; Carpenter *et al.*, 1987).

These three elements can be divided into two groups. *Tam1* and *Tam2* are comparable with the *Spm* maize elements, due to their homologous termini and a 3 bp target duplication. They also share homology to the *Tgm1* element of *Glycine max*

(Vodkin *et al.*, 1983). *Tam3* differs obviously from *Tam1* and *Tam2* but shows a homology to the terminal nucleotides from the *Ac* element of maize. They share the same length of target duplication (8 bp) while considering the *Tam3* insertion at the *niv* locus. Further similarity is shown in a common feature of excision modes, based on the absence of 1 bp at the axis of symmetry produced in the inverted duplications (Coen *et al.*, 1989). Other characterized elements in *Antirrhinum majus* are *Tam7* and *Tam9* (see also Table 1).

3.3. Repetitive Transposons in Plants

Higher plants usually have very large genomes when compared with genomes of other eukaryotes. This is due to an enormous amount of repetitive DNA in plant genomes. A large quantity of the repetitive DNA, especially the interspersed repetitive sequences, are suggested to be transposons or their remnants (Flavell, 1986). Some known retrotransposons such as *del2* elements of *Lilium* species (Leeton and Smyth, 1993) and *Bare-1* in *Hordeum vulgare* (Suoniemi *et al.*, 1996) can occupy up to 4% or 7% respectively of the genome alone (for retrotransposons see also section 3.4). Other middle or highly repetitive sequences include *Lyt1* from *Lycopersicon* species as well as *Mu* (see also sections 3.1 and 3.4), *tourist* (Bureau and Wessler, 1992) and *stowaway* elements (Bureau and Wessler, 1994), originally detected in maize. The latter two contain short terminal inverted repeats (TIR) but it is unlikely that they code for any protein gene. They are of small size (*tourist*: 113–343 bp; *stowaway*: 80–323 bp) and several family members are able to form hairpin structures. It is proposed that they are related to bacterial or animal *IS* because they possess analogous characteristics and structural similarities. However, they do not share any sequence homology with known *IS* elements. It is also possible that these elements represent a class of solo LTR sequences, because LTR often possess TIRs. The mode of transposition of these elements remains unclear; however, the presence of short terminal inverted repeats indicates a 'cut-and-paste' mechanism. To date, *tourist* elements have been found exclusively in more than 30 monocotyledonous plant species whereas *stowaway* transposons have been identified in monocots as well as dicots (around 20 plant species). There are two features which make these two transposon classes interesting for investigation and indicate further participation of such elements in the evolutionary processes. The first feature is their ability to form secondary structures and the secondly is the strong preference for defined target sites within the host genome. For instance, in *Hordeum vulgare* genes encoding thaumatin-like proteins, AT-rich elements have been found at the very proximal 3′-ends of genes serving as polyadenylation signals. *Stowaway* was also found in pseudogenes and in the 5′-regulatory region of genes, such as the regulatory sequences of the *ssu*-rubisco promoter of tomato (Bureau and Wessler, 1994). Thus, such transposable elements seem to be involved in the creation of functional gene cassettes as well as in the development of gene families. *Tourist* and *stowaway* also share a special feature with bacterial *IS*, which is unique among plant transposable elements; that is, they possess a target site specificity, being TAA for *tourist* and TA for *stowaway*. This makes them candidates for recombination events in plant genomes too.

3.4. Retrotransposons in Plants

Plant retrotransposons are mobile DNA sequences that spread over the genome through RNA intermediates. They represent the largest and most common class of mobile elements in plants. The occurrence of retrotransposons in a broad range of plant species has been reported, including *Zea mays* (Jones *et al.*, 1985; Vignols *et al.*, 1995; White *et al.*, 1994), *Arabidopsis thaliana* (Voytas and Ausubel, 1988; Konieczny *et al.*, 1990), *Hordeum vulgare* (Suoniemi *et al.*, 1996), *Triticum aestivum* (Harberd *et al.*, 1987), *Vicia* species (Pearce *et al.*, 1996), *Nicotiana* species (Grandbastien, 1992; Royo *et al.*, 1996), *Beta vulgaris* (Schmidt *et al.*, 1995) and *Oryza sativa* (Hirochika *et al.*, 1992). Hirochika and Hirochika (1993) screened 35 plant species for *Ty1/copia*-like retrotransposons. Sequences of the expected size were amplified from all these plant species and contained sequences with homology to reverse transcriptase and polymerase genes. Similar results were reported by Voytas *et al.* (1992), who identified *copia*-like retrotransposons in 64 plant species.

However, up to now only a few of these have been shown to be active in plants. For instance, among higher plant retrotransposons, the mobility has been shown for the tobacco retrotransposons *Tnt1* (Grandbastien *et al.*, 1989; Pouteau *et al.*, 1994), *Tto1* (Hirochika, 1993) and rice *TOS17* (Hirochika *et al.*, 1996).

Although we are currently just beginning to understand the action of retrotransposons in plants, it is apparent that they play an important role in plant genomes. By forming RNA intermediates that are reverse transcribed into DNA, retrotransposons proceed through cycles of duplication and amplification, ensuring that the copy number of these elements is high in the genome (10^4 to 10^5 copies per haploid genome) with only a few exceptions (e.g. some *Arabidopsis thaliana* retroelements; Konieczny *et al.*, 1991). It has been suggested that retroelements may be a driving force during recombination events and karyotypic changes, thus becoming one of the causes of somaclonal variation. This is evidenced by the strong induction of the transcriptional activity of *Tnt1* during protoplast isolation (Pouteau *et al.*, 1994, see also section 4). Large amounts of extrachromosomal forms of the tobacco *Tto1* were found in *in vitro* cultured tobacco cells but not in the plant material (Hirochika and Otsuki, 1995). In addition, Galliano *et al.* (1995) described a DNA sequence with features of a retrotransposon that stimulates DNA recombination in several plant species, which may lead to general recombination processes.

Furthermore, retrotransposons can incorporate and transmit a portion of a nuclear gene transcript (mRNA of the *mhal* locus in maize) within its genetic material (Jin and Bennetzen, 1994). This leads to a new proposal that transposable elements can function to transport host genetic material from one locus to another, a so-called transduction of genetic information.

4. Uses of Transposons for Mutagenesis and Identification of Genes

Molecular biology and genetic engineering are most important tools of present-day plant breeding. The role of genetic engineering is to identify and isolate plant

genes. The cloning of plant genes is complicated due to high complexity of plant genomes, which includes a high number of genes and a large quantity of repetitive DNA as compared to bacterial genomes. Therefore, it is advantageous to have molecular tools for 'labelling' and cloning of plant genes. Transposable elements are ideal molecular tools because they are: (a) mobile and most of them are able to insert into any locus of the plant genome and (b) able to switch off or alter the gene expression. At the beginning of the recombinant DNA technology era it was already recognized that mutant genes derived from transposon insertion could be isolated because they are 'tagged' by the transposable element. This further indicates that the sequences flanking the transposon integration site of such mutants are genes of great interest. 'Transposon tagging' can be used for the isolation of unknown genes by conferring a phenotype alteration of mutants. Usually, the tagging process includes the cloning of the known transposable element together with portions of the mutant allele from the plant DNA, followed by characterization of the sequences flanking the transposon (Walbot, 1992a). In order to identify the sequences, the wild type allele should be cloned and, if possible, used for complementation of the mutant in case of non-autonomous transposons. Another approach is the screening for mutant revertants exhibiting the normal phenotype. They should be investigated to verify the excision of the transposable element from the mutated locus (in case of autonomous or *trans*-activated non-autonomous transposons).

However, it is not always true that transposable elements integrate randomly to all parts of the genome. Transposition of different transposons often leads to the reintegration of the element very close to the excision site (Döring and Starlinger, 1984 for *En/Spm*; Greenblatt, 1984 for *Ac/Ds*; Schnable and Peterson, 1986 for *Cy*). A donor site-specific integration sequence has been described for two plant transposable elements, *tourist* (target site sequence is TAA) and *stowaway* (target sequence TA; Bureau and Wessler, 1994). In maize, some transposable elements were located in defined loci preferentially. Some of the original investigations of transposable elements described the occurrence of so-called 'hot spots' in plant genomes where insertions of transposable elements were very frequent. In addition, the induction of transposon-derived mutants by application of stress factors often led to a more or less defined spectrum of mutants (Coen and Carpenter, 1986 for *Tam3*; Nacken *et al.*, 1991 for *Tam1*). The activation of transposons during application of environmental stress is known for DNA-based elements as well as for retrotransposons. Different factors can influence both the amount of a transposable element's transcription and its mobility. DNA-damaging agents such as irradiation or UV light, that are known to induce genomic stress situations, activate transposable elements in yeast (Bradshaw and McEntee, 1989) and in plants (Walbot, 1988, 1992c). Another abiotic factor, temperature, also influenced the transposition frequency in plants. On the one hand a decrease in temperature can cause more frequent transposition, e.g. a 1000-fold increase in the case of the *Antirrhinum majus* element *Tam3* (Carpenter *et al.*, 1987) while on the other hand an increase in temperature can likewise result in higher mobility of the *Ac2* element (Osterman, 1991).

Tissue culture conditions and isolation of single plant cells (protoplasts) are among the most important biotic factors that influence the activity of many trans-

posable elements (Grandbastien, 1992). Although clear evidence for single factors during *in vitro* culture was not found, it was clear that the process of tissue culture leads to an increase in transposon action in several plant species, such as for the transposons *Tnt1* (Pouteau *et al.*, 1994) and *Tto1* (Hirochika and Otsuki, 1995) of tobacco and maize *Mu*. It was also found that the synthesis of transcripts was strongly enhanced by protoplast isolation and tissue culture when compared with the transcription level *in planta*. In combination with factors including bacterial elicitors, significantly higher transcription levels of *Tnt1* were detected as compared to controls (Pouteau *et al.*, 1994).

Activity changes that transposable elements undergo can be studied in more detail by introducing them into heterologous hosts (see also section 4.2). Tissue culture after genetic transformation enhanced somatic activities which become visible and measurable, as a result of introduced elements that are novel to the host and present only in single or low copy numbers. Using this approach, Lucas *et al.* (1995) found that *Tnt1* was transposing during *in vitro* regeneration of transformed *Arabidopsis thaliana* but no longer in the T_2 and T_3 generations. These results revealed that retrotransposons can be very active during somatic processes in plant cells and are able to induce somatic changes frequently.

Figure 2 shows the somatic mobility of members of the *Ac/Ds* family following introduction into *Datura innoxia*. It shows that active *Ac* elements retain high levels of transposition for a long time under tissue culture conditions. This leads to somaclones with a continous and variable *Ac* integration pattern of the transposable

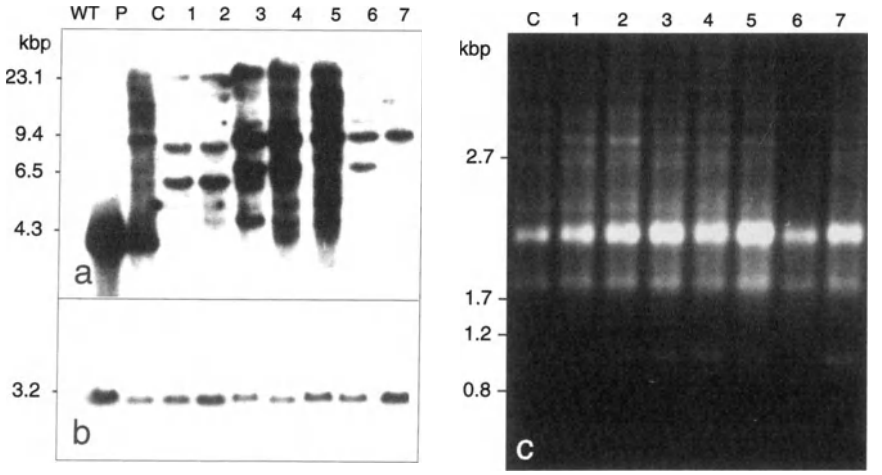

Figure 2. Somatic transposition of the *Ac* element in tissue culture of transgenic *Datura innoxia* Mill. Protoplasts were transformed with a construct similar to that shown in Figure 3a. **(a)** When using an *Ac*-specific hybridization probe, Southern analysis of seven different *in-vitro*-cultivated plants (lanes 1–7) and the initial callus (lane C) of one transgenic clone show both a continued somatic excision/reintegration and an amplification of the transposable element in several plants. **(b)** This somatic excision/reintegration has no consequences on vector sequences, neighbouring the *Ac* at the construct integration site, as shown for the *nptII* gene. **(c)** PCR-based fingerprinting with different primers revealed that no major changes in the genome occurred. In this case $(GATA)_4$ was used for amplification. WT: Wild type plant DNA, P: plasmid (control) DNA

element (Fig. 2a; Schmidt-Rogge *et al.*, 1994). These changes do not affect parts of the vector used for the introduction of the element (Fig. 2b) and seem to have no major influence on the whole genome as revealed by PCR-based fingerprinting with different primers (Fig. 2c).

Activity changes of transposons are often – if not always – correlated with changes of the methylation pattern of the transposon DNA. As described for the *Ac* element and other transposons too (e.g. *En/Spm*: Banks *et al.*, 1988; *Mu*: Bennetzen, 1987), the methylation state drastically influences the activity of the transposable element (Schwartz and Dennis, 1986). The possible regulation mechanisms are methylations within the transposase promoter/coding region that decrease or prevent the transcription (Chomet *et al.*, 1987), and C-methylations in critical positions of the subterminal repeat regions (Fig. 1) of *Ac* or *Ds*, that can lead to a strong reduction in excision frequency as well as to an increased binding of the transposase to this subterminal regions. Furthermore, the effect of changes in the methylation pattern of the host DNA flanking the transposable element on the mobility of transposons has to be discussed, because it is known to affect gene expression in general (Pröls and Meyer, 1992).

Transposon tagging was first applied in plants containing well-characterized transposable elements, e.g. *Zea mays* and *Antirrhinum majus*. By transposon tagging, molecular cloning of the first mutant alleles in these two plant species dates back more than 15 years. The isolation of new genes and the characterization of transposons at the molecular level progressed simultaneously.

4.1. Transposon Tagging in Native Hosts

As mentioned earlier, most of the genes isolated by transposon tagging come from *Zea mays* and *Antirrhinum majus*. Up to date, approximately 40 different loci have been identified in maize (Table 2), and more than 10 loci in snapdragon. The *waxy (wx)* locus (Shure *et al.*, 1983) and the *opaque2 (o2)* locus (Hartings *et al.*, 1989; Michel *et al.*, 1995) have been the most intensively studied loci in *Zea mays*. Several transposable elements that create different alleles were isolated from these two loci (Table 2). With a few exceptions, these genes were tagged by DNA-based transposable elements.

Transposons can either integrate into genes and cause disruption or activity modulation of the appropriate gene (forward mutation) or they can jump out of a locus leading to partial or full recovery of gene activity (somatic or germinal reversion). The majority of genes isolated by transposon tagging were found after excision from a mutated locus and, thus, reversion to the wild type. Somatic excision was described for several transposable elements in different plant species. It can result in a dramatic phenotype when it occurs frequently on loci, for instance, responsible for flower pigmentation (loci of the anthocyanin pathway). In such plants, somatic unstable lines are characterized by candy-striped or purple-flecked pigmentation of the flower petals, a desirable trait in cut flowers. Successful germinal excision of a transposable element from a mutant locus may lead to the recovery of the wild type or a quasi wild type phenotype. Such revertants can

serve as a proof that the mutated/reverted locus contains the gene responsible for the expression of the mutant phenotype.

The occurrence of different variegation patterns in maize, especially in so-called Indian maize, was the first indication of unstable mutations of unknown origin. McClintock (1948) demonstrate that these phenotypic phenomenons could be correlated with the occurrence of 'jumping genes'. Later physical evidence showed the integration of transposons at about four dozen gene loci responsible for maize kernel coloration, storage protein and starch synthesis. These days many of these loci are cloned from maize lines containing different transposon-harbouring alleles (mostly *Ac/Ds*) by transposon tagging and the wild type genes are extensivly characterized (Fedoroff, 1983; Nevers *et al.*, 1986; Maize Data Base; personal communication, E. Coe). For a detailed listing of the identified genes with the transposon tagging method in maize see Table 2.

In *Antirrhinum majus*, several loci were analysed that were involved in flower pigmentation and flower morphology employing *Tam* elements. De Vries (1910) was the first to analyse the inheritance of variegated flower colour. Wild type plants were characterized by full-colour red flowers, whereas mutant plants had ivory-coloured flowers with red stripes or spots. The earlier the changes occur in flower development, the larger the sector of flower colour. A complete red colour revertant progeny was also observed when a change occurred in gametic tissue. With the discovery of transposable elements, these changes could be explained as a result of insertion of transposable elements into loci required for pigment synthesis, producing recessive (no colour) alleles. The *nivea (niv)* locus, for instance, encodes the enzyme chalcone synthase and is required for red anthocyanin pigment production in flowers, leaves and stems. Excision events lead to variegated or wild type coloured flowers. An interesting feature of these unstable alleles is that they can generate a series of derivative alleles conferring different intensities in pigmentation. This is due to structural alterations within the transposable element or changes of the position of these elements within the gene, leading to modulation of gene expression. Another locus called *pallida (pal)* encodes the NADPH-dependent dihydroflavonol-4-reductase, which is involved in the red anthocyanin pigment synthesis too. In alleles where *Tam3* was positioned (*palrec2*), a variegated phenotype was described in which red spots or sectors were seen on an ivory background. This element was also used for the cloning of this locus (Martin *et al.*, 1985). One example for a homeotic gene involved in flower development and identified by transposon tagging with *Tam7* is *deficiens* conferring a homeotic change, that is sepals grow instead of petals and stigmata tissue in place of stamens (Sommer *et al.*, 1990). Other examples of genes involved in the regulation of flower development and isolated by the help of transposable elements, are *floricaula* tagged by *Tam3* (Coen *et al.*, 1990) and *globosa* tagged by *Tam7/Tam9*.

High copy numbers of transposable elements (such as the retrotransposons *Tnt1* from tobacco and the *Mu* family from maize, but also *Ds* or *dSpm*) in combination with a high transposition activity increase the chances for identifying new genes of interest. However, the isolation of genes tagged by high copy number transposable

elements is much more difficult, because the detection and isolation of a single integration event cosegregating with the locus responsible for the mutation is not easily achieved. In order to rationalize the cloning of such genomic sequences, Earp *et al.* (1990) used the inverse polymerase chain reaction method to amplify sequences flanking the transposed elements. Using this method in a maize line with multiple *Spm* elements, they successfully cloned a specific *Spm* element.

Grandbastien *et al.* (1989) used the targeted transposon tagging to show the action of an endogenous transposon and to clone it from tobacco. In tobacco tissue cultures, through *in vitro* selection, spontaneous mutants of the *nia* locus (Vaucheret *et al.*, 1989) were selected and the retotransposon *Tnt1* was isolated from such a *nia* mutant after its insertion into the nitrate reductase structural gene (Grandbastien *et al.*, 1989). *Tnt1* has a length of 5.3 kbp and is flanked by 610 bp long terminal inverted repeats. It contains a single open reading frame. Sequence comparison shows a high homology with the *copia* retrotransposons of *Drosophila melanogaster* and extensive homology was found with reverse transcriptase and polymerase gene conserved domains. This led to the conclusion that *Tnt1* possessed all necessary functions for its autonomous transposition (Grandbastien, 1992). By this method the identification of the retrotransposon *Tnt1* as well as its insertion into the *nia* locus could be achieved. The frequency for the somatic integration was found to be $1–3 \times 10^{-5}$ in this experiment.

4.2. Introduction of Transposons into Heterologous Hosts

The cloning and molecular characterization of transposable elements from *Zea mays* (namely *Ac*: Fedoroff *et al.*, 1983; Behrens *et al.* 1984; Pohlman *et al.*, 1984; Müller-Neumann *et al.*, 1984) and *Antirrhinum majus* (Bonas *et al.*, 1984; Upadhyaya *et al.*, 1985) were a major breakthrough in plant molecular genetics in the beginning of the 1980s. These studies led to three kinds of results:

(a) a detailed structural and functional analysis of transposable elements was possible now (see sections 3.1 and 3.2);
(b) they could now be used as molecular tools for the isolation of mutant loci from especially maize and snapdragon plants, already having large collections of transposon-derived mutants (see section 4.1); and
(c) the use of well-investigated transposable elements for transposon tagging in other plant species, lacking good characterized transposable elements.

Besides subtractive cloning (Straus and Ausubel, 1990), DDRT–PCR (Liang and Pardee, 1992; Utans *et al.*, 1994) and T-DNA insertional mutagenesis (Feldman, 1991; Koncz *et al.*, 1992), transposon tagging has continued to become more important in model plants such as *Nicotiana tabacum*, *Arabidopsis thaliana* and agronomically important plants. Today, it opens the way to clone and identify plant genes of scientific or agricultural importance by random and targeted tagging strategies. All known transformation procedures can be used for the introduction of transposable elements into heterologous hosts.

Transposable elements from three plant species, namely *Ac/Ds* from maize (Baker *et al.*, 1986; Table 3), *En/Spm* from maize (Masson and Fedoroff, 1989; Cardon *et al.*, 1993; Table 3), *Mu* from maize (Zhang and Sommerville, 1987), *Tam3* from snapdragon (Martin *et al.*, 1989; Haring *et al.*, 1991), *Tnt1* from tobacco (Pouteau *et al.*, 1994; Lucas *et al.*, 1995) and *Tto1* from tobacco (Hirochika *et al.*, 1996) were tested for their ability to transpose in other plants. Several researchers showed that they, with the exception of the retrotransposon *Mu* (Delguidice *et al.*, 1991), were able to transpose in heterologous host plants. Although big differences were reported in the transposition frequency in different plant species, the transposition mechanism seemed to be the same in all plants and so are the host factors that are required for the transpositional process in addition to the element-specific functional transposase protein.

The most frequently used transposons for transposon tagging belong to the *Ac/Ds* family. Baker *et al.* (1986) described for the first time the transposition of the *Ac* element in a heterologous host. Meanwhile *Ac* and *Ds* elements have been shown active in more than 20 different plant species including *Nicotiana tabacum* (e.g. Baker *et al.*, 1986; Masterson *et al.*, 1989; Jones *et al.*, 1993), *Arabidopsis thaliana* (e.g. van Sluys *et al.*, 1987; Schmidt and Willmitzer, 1989; Dean *et al.*, 1992), *Daucus carota* (van Sluys and Tempe, 1989), *Glycine* species (Zhang and Sommerville, 1987; Zhou and Atherly, 1990), *Lycopersicon esculentum* (e.g. Yoder *et al.*, 1988; Jones *et al.*, 1990; Hille *et al.*, 1993), *Oryza sativa* (e.g. Murai *et al.*, 1991; Shimamoto *et al.*, 1993) and *Solanum tuberosum* (e.g. Knapp *et al.*, 1988). Excision assays were used in order to establish a transposon tagging

Table 3. Genes identified by transposon tagging in heterologous plants

Plant species	Gene/locus	Transposon	Reference
Arabidipsis thaliana	*alb3*	*Ds*	Long *et al.*, 1993
	dif, b-p2-1, eleAc	*Ac*	Bhatt *et al.*, 1996
	drl1	*Ds*	Bancroft *et al.*, 1993
	fae1	*Ac*	James *et al.*, 1995
	lrp1	*Ds*	Smith and Fedoroff, 1995
	ms2	*En*	Aarts *et al.*, 1993
	prl	*Ac/Ds*	Springer *et al.*, 1995
	ssr16	*Ds*	Tsugeki *et al.*, 1996
	tiny	*Ds*	Wilson *et al.*, 1996
	*303, 396, 343, 529**	*Ac/Ds*	Altmann *et al.*, 1995
Linum usitatissimum	*16*	*Ac*	Lawrence *et al.*, 1995
Lycopersicon esculentum	*cf-9*	*Ds*	Jones *et al.*, 1994
	feebly	*Ac/Ds*	van der Biezen *et al.*, 1996
Nicotiana plumbaginifolia	*aba2*	*Ac*	Marin *et al.*, 1996
Nicotiana tabacum	*n*	*Ac*	Dinesh-Kumar *et al.*, 1995
Petunia hybrida	*ph6*	*Ac*	Chuck *et al.*, 1993

*Putatively tagged.

system and to monitor transposition events in the plant species of choice. This means that a marker or a reporter gene cassette, which is normally active in plants, was physically disrupted by the insertion of the transposon (mostly insertion in the untranslated leader region of the gene). After transformation of plant cells with these constructs, the marker/reporter gene cassette is restored upon excision of the transposable element resulting in desired phenotypes. Various excision markers have been used, for instance the *aphIII* gene conferring resistance to aminoglycoside antibiotics (Baker *et al.*, 1987) or the *spt* gene for spectinomycin resistance (Jones *et al.*, 1989) and the *uidA* gene producing β-glucuronidase (Jefferson, 1987). This enzyme can be used as a reporter or in quantitative colorimetric assays (Houba-Herin *et al.*, 1990).

Many different strategies and transposon tagging systems have been developed in the past, based on the use of autonomous as well as non-autonomous transposable elements. Figure 3 demonstrates two principal strategies for the establishment of transposon tagging using different members from the *Ac/Ds* transposon family. In Figure 3a the transposon, inserted between the promoter and the coding sequence of the marker/reporter gene, is an autonomous element (*Ac*). It catalyses its own excision (single component system). A non-autonomous (artificial *Ds*)

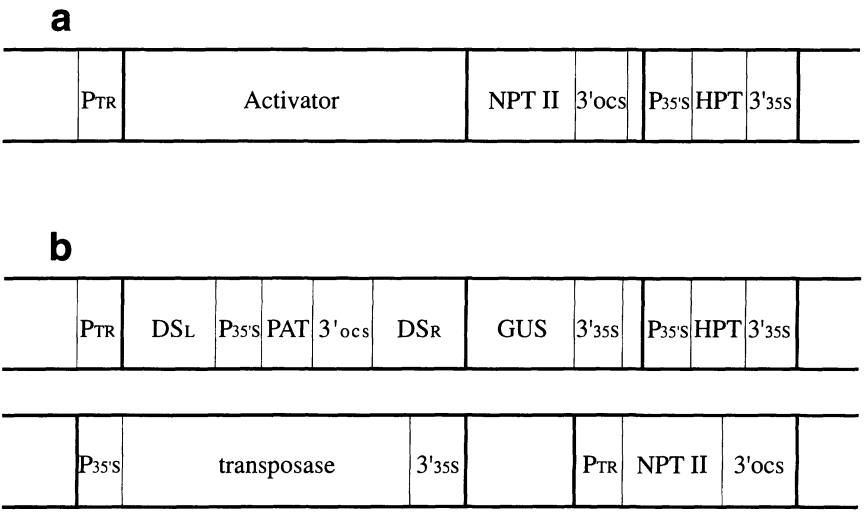

Figure 3. Schematic draw of a single component **(a)** and a two-component **(b)** transposon tagging system, based on the use of *Ac/Ds* elements from *Zea mays*. **(a)** The *Ac* element (*Activator*) is inserted in the untranslated leader sequence between the TR promoter (P_{TR}) and the *aphIII* gene (NPTII-3′ ocs). This allows monitoring of the excision of the element, whereas the plant hygromycin resistance cassette ($P_{35'S}$-HPT-$3'_{35S}$) can be used for determination of transformation efficiency and selection; **(b)** A two-component system requires both a non-autonomous *Ds* element (DS_L-$P_{35'S}$-PAT-$3'_{35S}$-DS_R, upper graph) disrupting a plant gene cassette (P_{TR}-GUS-$3'_{35S}$, GUS excision assay) and a source of transposase (P_{35S}-transposase-$3'_{35S}$, graph at the bottom). The HPT gene cassette (upper graph) and the NPTII gene cassette (graph at the bottom) can be used as markers for selection and in crosses, whereas the PAT gene cassette can serve as transposon reintegration marker and for selection of the progency harbouring the *Ds*

element requires a second component, that provides the element-specific transposase (Fig. 3b). Two-component systems can be created by either successive transformations and segregation analysis of the double transformant or by crossing *Ds*-lines with independently transformed transposase lines. In the F_1 of crosses the transposase can act *in trans* and thus mobilize *Ds* elements. In the F_2 of the crosses the transposase and eventually also the remaining parts of the construct segregate from the *Ds* element. The F_2 progeny can be used for the isolation of (putatively tagged) mutants. The use of additional marker/reporter genes is recommended to determine the cause of absence of the transposase source in two-component systems (Fedoroff and Smith, 1993) as well as for measuring the reintegration frequency of the transposable element and the transformation efficiency (Schmidt-Rogge *et al.*, 1994) or for selection of the progeny. These measurements allow the determination of the transposition frequency as well as the prediction of new mutations and a critical estimation of the used transposon tagging system in the favourable plant species.

Since the first publication of transposition of *Ac* in tobacco, it took 7 years before the first gene was tagged in the heterologous host *Petunia hybrida*. The introduced *Ac* element jumped into *Ph6*, one of several loci that modify anthocyanin pigmentation in petunia flowers. This new mutation was unstable and reverted to wild type in somatic and germinal tissues. It was also shown that a recovery of the wild type is correlated with restoration of a wild type-sized DNA fragment (Chuck *et al.*, 1993). As listed in Table 3, so far more than 10 genes have been identified by transposon tagging in *Arabidopsis thaliana* by application of *Ac* or *Ds* with only one exception where the *ms2* gene was tagged by an *En* element (Aarts *et al.*, 1993). Also in other plant species, genes have been identified using transposon tagging; these include *Linum usitatissimum* (one gene), *Lycopersicon esculentum* (two), *Nicotiana tabacum* (one) and *Nicotiana plumbaginifolia* (one) (Table 3). These examples show that transposon tagging is a labour-intensive but powerful tool for the identification of genes in many plant species, limited only by transformation and/or tissue culture techniques. This might be the reason why until now there is no report of genes tagged by the use of heterologous transposons in monocotyledonous plant species. In *Arabidopsis thaliana* (Fedoroff and Smith, 1993; De Long *et al.*, 1993; Altmann *et al.*, 1995) and *Lycopersicon esculentum* (Jones *et al.*, 1994) large stocks of transposon-containing plant material exist thus the genome was statistically saturated with foreign transposable elements. Therefore in principle it should be possible to tag any gene of interest in these two species.

5. Conclusions

In plants DNA-based transposable elements as well as retrotransposons have been found. Both types are suggested to play an important role in mutational changes within plant genomes ranging from insertions, deletions, and duplications to translocations and chromosome abberations. Otherwise they are able to alter the

expression of genes, to transport genes from one location to another and to mediate rearrangements within chromosomes and genomes, thus being one of the principles responsible for evolutionary changes within genomes. The activity of many transposons is influenced by changes of abiotic as well as biotic factors, such as irradiation, temperature and *in vitro* culture for instance. Therefore, efficient mutation strategies can be developed by modulation of the activity of individual transposons in their host plants by changes of abiotic factors or application of tissue culture.

Transposons are powerful tools in the isolation of unknown genes. Different methods have been used for gene tagging by the help of transposons in *Zea mays* and *Antirrhinum majus*. Altogether more than 50 loci have been identified by transposon tagging in *Zea mays* and *Antirrhinum majus* to date. Transposable elements, especially *Activator/Dissociation*, were also used for the identification of genes in plant species, where transposable elements are unknown or only poorly characterized. About 20 genes in six different plant species have been identified by transposon tagging in heterologous plant species to date.

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29. Detection of Dwarf Somaclones of Banana Cultivars (*Musa*) by RAPD Markers

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Introduction

Banana is one of the most important subtropical fruit crops. The banana is believed to have originated in Southeast Asia. Since the eleventh century, banana has spread widely throughout the tropics and is considered as one of the most important agricultural products in many developing countries. It is currently grown in South America as far as Bolivia and most of Brazil. In Africa, banana is grown from the Sahara to Tanzania in the east, and from the Ivory Coast to Zaire in the west (FAO Publications, 1990).

Somaclonal Variation in Banana (Musa)

In-vitro shoot-tip culture is a basic technique used for banana (*Musa*) propagation, medium-term conservation and germplasm exchange (Vuylsteke, 1989). With the increased use of tissue culture techniques, somaclones were detected among field-established banana plants (Reuveni and Israeli, 1990). A description of banana somaclones, their occurrence and the stage at which they can be detected was reported by Israeli *et al.* (1991). The nature of the changes, genetic or epigenetic, occurring in banana somaclones have yet to be established.

Many banana somaclones do not perform well in the field and result in an economic loss to farmers. The detection and elimination of somaclones represent essential steps in the application of the *in-vitro* technique for propagation, germplasm exchange and conservation. The maintenance of genetic stability of somaclones is of utmost importance.

Dwarf off-types are the most common variation in Cavendish bananas, and account for about 80% of the total variants (Reuveni and Israeli, 1990). The dwarf undesired variants are difficult to detect in the nursery. Therefore, it is highly desirable to have a reliable technique for their detection.

Morphological Markers

The morphological-based characterization of dwarf somaclones during *in-vitro* culture is limited. It was possible to detect extra dwarf variants, which were formed at high incidence when 'Nathan' (a selection of dwarf Cavendish) was micropropagated (Israeli *et al.*, 1991). Dwarf and normal plantlets of 'Williams'

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bananas responded differently to gibberellic acid (GA3). In normal plantlets, GA3 elongated internodes and suppressed leaf production, while dwarf plantlets were much less sensitive to GA3 (Reuveni, 1990). The earliest *in-vitro* stage to detect dwarfs was at the end of their growth in the nursery. The differences in height, distance between petioles, and leaf size could be distinguished very well (Israeli *et al.*, 1991). The most promising selection criteria of somaclones were petiole length, lamina length and petiole to lamina length ratio (Smith and Hamill, 1993). Detection was effective when plants were growing vigorously and uniformly. The effect of GA3 treatment was also tested on 'Grand Nain' *in-vitro* plants during acclimatization (Cote *et al.*, 1993). This treatment resulted in faster extension in normal compared to dwarf plants. The disadvantage of these methods, that are based on early morphological identification, is that they are prone to environmental effects. They are sufficient to detect undesired dwarfs in a commercial multiplication operation, but are not sufficient when *in-vitro* germplasm conservation is considered.

Molecular Markers

Earlier, attempts were made to distinguish banana cultivars by using biochemical markers (Novak, 1992). Isozyme polymorphisms were reliable markers for making distinction among *Musa* clones of interspecific hybrids of *M. acuminata* and *M. balbisiana.* However, no differences were observed among different triploid banana clones and their somaclones. Recently, Kaemmer *et al.* (1992) reported that DNA oligonucleotide and amplification fingerprinting were successfully used to detect genetic polymorphism in representative cultivars of the genus *Musa.* Different cultivars of the Cavendish banana group and an induced mutant of the 'Grand Nain', belonging to the same group, could be recognized by the random amplified polymorphic DNA (RAPD) technique. Similarly, Howell *et al.* (1994) used RAPD to identify nine genotypes of *Musa* representing AA, AAA, AAB, ABB, and BB genomes. The pattern of variation observed following the application of multivariate analyses to the RAPDs banding data was very similar to the pattern of variation defined using morphological characters. Also, this pattern of variation was used to assign *Musa* material into the different genome classes. Bhat *et al.* (1995) reported the success of using of RAPD in 57 *Musa* cultivars to unambiguously identify them and construct a phenogram. They also used a restriction fragment length polymorphism (RFLP) technique that resulted in a phenogram comparable to the one obtained with RAPD analysis. RAPD analysis is a cost- and time-effective alternative to the RFLP analysis. However, many researchers questioned the reproducibility of RAPD analyses conducted among different laboratories. Penner *et al.* (1993) reported that DNA fragments amplified by five primers were shown to be reproducibly polymorphic between two oat cultivars (within their laboratory). They were also tested in six other laboratories in North America. The reproducibility of results was primarily affected by the performance of thermocyclers. Reproducible results could be obtained after individual fine-tuning of the temperature profiles, especially the annealing temperature.

We studied the possibility of using the RAPD technique for detecting dwarf somaclones of three cultivars of the Cavendish subgroup of bananas. Except for other characteristics, the cultivars differed in their stature: 'Williams' being the tallest and 'Nathan' (a selection of 'Dwarf Cavendish') the shortest.

Materials and Methods

Plant Materials (Cavendish Banana)

Suckers of normal and dwarf somaclones found in the field among *in-vitro*-propagated 'Williams', 'Grand Nain' and 'Nathan' bananas were transplanted in the experimental plot. It was verified phenotypically that dwarfism transmitted to suckers (Reuveni and Israeli, 1990). Suckers from such stable plants were transplanted and grown in 50-litre containers in the greenhouse at the Volcani Center. Samples were taken from different plants and on different dates from the middle part of the fully expanded leaves.

DNA Extraction and PCR Amplification

Fresh leaves of banana plants were stored at –70°C up to several months prior to DNA extraction. Total genomic DNA was extracted by using a modified CTAB DNA extraction procedure developed by Gawel and Jarret (1991). The DNA was quantified with fluorometer (Hoffer Scientific Inc.). Target DNA sequences were amplified by PCR in programmable thermocycler (M&J Inc.). PCR conditions were as described by Williams *et al.* (1990) except that 20 ng DNA was used per reaction. Taq polymerase was purchased from Promega Inc. Each reaction tube contained a single 10mer random oligonucleotide purchased from Operon Technologies Inc. One hundred different primers (Kits A–E) were tested at least three times for all the primers that successfully generated bands in both normal and dwarf mutants. PCR amplification products were analyzed by 2% agarose/TBE gel electrophoresis (Sambrook *et al.*, 1989), and stained with ethidium bromide. Degree of polymorphism (DP) was defined as $(1 - BS) \times 100 = DP$, whereas BS was the 'band sharing' calculated according to Vainstein *et al.* (1991).

Results and Discussion

One hundred different primers were tested. Figure 1 shows typical DNA fingerprints generated by PCR of normal and dwarf somaclones of 'Williams', 'Grand Nain' and 'Nathan'. This picture does not reveal any significant polymorphism. Figure 2 indicates typical RAPD markers obtained using five different primers and a comparison between cv. 'Williams' and its dwarf somaclone. Polymorphism is clearly observed between D1 and D2. The random amplified

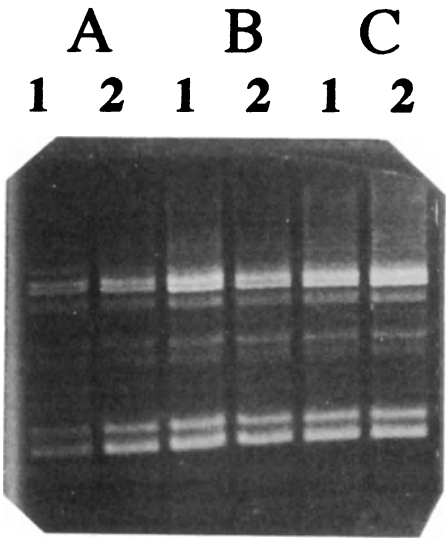

Figure 1. RAPD analysis of normal (1) and *in-vitro* dwarf somaclones (2) of 'Williams' (A), 'Grand Nain' (B), and 'Nathan' (C), using C18 primer

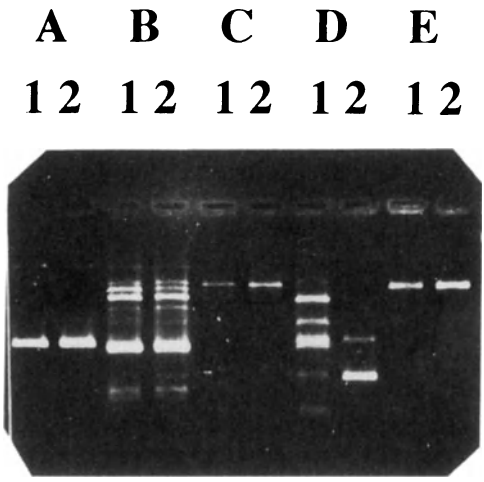

Figure 2. RAPD analysis of normal (1) and dwarf somaclone (2) banana cv. 'Williams'. A, B, C, D, and E, represents primers C4, C5, C8, C11, and C14 respectively

Table 1. Random amplified DNA polymorphism between three banana cultivars and their dwarf variants and between three normal banana cultivars

Cultivars	Polymorphism (%)	Number of primers	Number of bands
'Williams' X dwarf	7.16	73	335
'Grand Nain' X dwarf	6.29	56	254
'Nathan' X dwarf	3.20	61	281
'Williams' X 'Grand Nain'	0.82	58	266
'Williams' X 'Nathan'	0.53	67	312
'Grand Nain' X 'Nathan'	1.25	69	278

DNA polymorphism between the three banana cultivars and their dwarf variants is presented in Table 1. Between 56 and 73 primers (depending on the cultivar) successfully generated reproducible RAPD markers, at least three times using different DNA extraction batches. The other primers did not produce reproducible RAPD markers in normal or dwarf plants. All the primers that established a polymorphic marker, generated at least one band in the normal and dwarf somaclones. The polymorphic band was either present or absent in the normal cultivar as compared to the dwarf. Since the procedure depends upon a random primer, DNA amplification of polymorphic marker could be the result of either deletion or insertion of DNA (Baird *et al.*, 1992). These types of mutational event have been proposed to take place in plant tissue cultures, and are responsible for somaclonal variation (Evans, 1989). The calculated percentage of polymorphism was 7.16%, 6.29% and 3.20% for 'Williams', 'Grand Nain', and 'Nathan', respectively. These results indicate that extensive genomic rearrangements took place during the *in-vitro* propagation of the plants. Remarkably, the calculated percentage of polymorphism determined by comparing the three normal cultivars against each other, revealed lower values then those observed for each cultivar and its *in-vitro* somaclonal dwarf variant (Table 1).

Recently, Valles *et al.* (1993) demonstrated the usefulness of RAPD markers in showing genetic stability of meadow fescue (*Festuca pratensis* Huds.) plants regenerated from suspension cultures and protoplasts.

Conclusions

We have demonstrated that RAPD markers are useful to detect genetic instability of *in-vitro*-propagated *Musa.* Likewise other techniques such as RFLP, and more recently competitive PCR techniques (Baurens *et al.*, 1996), may be useful to detect genetic instability. The latter was used to assay copy number of repetitive elements in banana, enabling the authors to distinguish between two banana cultivars. The information obtained may be used to isolate specific DNA probes. The future development of automatic sampling of plant materials, followed by DNA hybridization with specific DNA probes, may serve as a tool for preselection of

genetically stable banana cultivars in the nursery. This development may have major economic value to the banana industry, because genetically uniform plants are so important in commercial plantations.

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