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Editors

Transgenic Crop Plants

Volume 1: Principles and Development

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Preface

Transgenic Plants – known also as Biotech Plants, Genetically Engineered Plants, or Genetically Modified Plants – have emerged amazingly fast as a boon for science and society. They have already played and will continue to play a significant role in agriculture, medicine, ecology, and environment. The increasing demands for food, feed, fuel, fiber, furniture, perfumes, minerals, vitamins, antibiotics, narcotics, and many health-related drugs and chemicals necessitate development and cultivation of transgenic plants with augmented or suppressed trait(s). From a single transgenic plant (Flavr Savr^{TM} tomato with a longer shelf-life) introduced for commercialization in 1994, we have now 13 transgenic crops covering 800 million hectares in 25 countries of six continents. Interestingly, the 13.3 million farmers growing transgenic crops globally include 12.3 million (90%) small and resource-poor farmers from 12 developing countries. Increasing popularity of transgenic plants is well evidenced from an annual increase of about 10% measured in hectares but actually of 15% in "trait hectares." Considering the urgent requirement of transgenic plants and wide acceptance by the farmers, research on transgenic plants is now being conducted on 57 crops in 63 countries. Transgenic plants have been developed in over 100 plant species and they are going to cover the fields, orchards, plantations, forests, and even the seas in the near future. These plants have been tailored with incorporation of useful alien genes for several desirable traits including many with "stacked traits" and also with silencing of genes controlling some undesirable traits.

Development, applications, and socio-political implications of transgenic plants are immensely important fields now in education, research, and industries. Plant transgenics has deservedly been included in the course curricula in most, if not all, leading universities and academic institutes all over the world, and therefore reference books on transgenic plants with a classroom approach are essential for teaching, research, and extension. There are some elegant reviews on the transgenic plants or plant groups (including a 10-volume series "Compendium of Transgenic Crop Plants" edited by two of the present team of editors C. Kole and T.C. Hall published by Wiley-Blackwell in 2008) and on many individual tools and techniques of genetic transformation in plants. All these reviews could surely serve well

the purpose for individual crop plants or particular methodologies. Since transgenic plant development and utilization is studied, taught, and practiced by students, teachers, and scientists of over a dozen disciplines under basic science, agriculture, medicine, and humanities at public and private sectors, introductory reference books with lucid deliberations on the concepts, tools, and strategies to develop and utilize transgenic plants and their global impacts could be highly useful for a broad section of readers.

Deployment of transgenic crop plants are discussed, debated, regulated, and sponsored by people of diverse layers of the society including social activists, policy makers, and the staff of regulatory and funding agencies. They also require lucid deliberations on the deployment, regulations, and legal implications of practicing plant transgenics. More importantly, depiction of the positive and realistic picture of the transgenic plants should and could facilitate mitigation of the negative propaganda against transgenic plants and thereby reinforce moral and financial support from all individuals and platforms of the society. Global population is increasing annually by 70 million and is estimated to grow up to 8 billion by 2025. This huge population, particularly its large section from the developing countries, will suffer because of hunger, malnutrition, and chemical pollution unless we produce more and more transgenic plants particularly with stacked traits. Compulsion to meet the requirements of this growing population on earth and the proven innocuous nature of transgenic plants tested and testified for the last 13 years could substantiate the imperative necessity of embracing transgenics.

Traditional and molecular breeding practiced over the last century provided enormous number of improved varieties in economic crops and trees including wheat and rice varieties that fostered the "green revolution." However, these crop improvement tools depend solely on the desirable genes available naturally, creatable by mutation in a particular economic species or their shuffling for desired recombinations. Transgenic breeding opened a novel avenue to incorporate useful alien genes not only from other cross-incompatible species and genera of the plant kingdom, but also from members of the prokaryotes including bacteria, fungi, and viruses, and even from higher animals including mice and humans. An array of plant genetic engineering achievements starting from developing insect resistance transforming with the *cry* genes in cotton from the bacteria *Bacillus thuringiensis* to the present-day molecular pharming expressing the *interferon-α* gene from human in tobacco evidence for this pan-specific gene transfer.

Human and animal safety is another general concern related to transgenic food or feed. However, there is no reliable scientific documentation of these health hazards even after 13 years of cultivation of transgenic plants and consumption of about one trillion meals containing transgenic ingredients. Utilization of transgenic plants has reduced the pesticide applications by 359,000 tons that would otherwise affect human and animal health besides causing air, water, and soil pollution and also mitigated the chance of consumption of dead microbes and insects along with foods or feeds.

Gene flow from transgenic crop species to their cross-compatible wild relatives is a genuine concern and therefore required testing of a transgenic crop plant before

deployment followed by a comprehensive survey of the area for the presence of interfertile wild and weedy plants before introduction of a transgenic crop is being seriously conducted.

Addition of novel genotypes with transgenes in the germplasms is increasing the biological diversity rather than depleting it. Use of the genetically engineered plants has also eliminated greenhouse gas emission of 10 million metric tons through fuel savings. In fact, 1.8 billion liters of diesel has been saved because of reduced tillage and plowing owing only to herbicide-resistant transgenic crops. Many transgenics are now being used for soil reclamation. Above all, cultivation of transgenic crops has returned $44 billion of net income to the farmers. Perhaps these are the reasons that 25 Nobel Laureates and 3,000-plus eminent scientists appreciated the merits and safety and also endorsed transgenic crops as a powerful and safe way to improve agriculture and environment besides the safety of genetically modified foods. Many international and national organizations have also endorsed health and environmental safety of transgenic plants; these include Royal Society (UK), National Academy of Sciences (USA), World Health Organization, Food and Agriculture Organization (UN), European Commission, French Academy of Medicine, and American Medical Association, to name a few.

Production, contributions, and socio-political implications of biotech plants are naturally important disciplines now in education, research, and industries and therefore introductory reference books are required for students, scientists, industries, and also for social activists and policy makers. The two book volumes on "Transgenic Crop Plants" will hopefully fill this gap. These two book volumes have several unique features that deserve mention. The outlines of the chapters for these two books are formulated to address the requirements of a broad section of readers. Students and scholars of all levels will obtain a lot of valuable reading material required for their courses and researches. Scientists will get information on concepts, strategies, and clues useful for their researches. Seed companies and industries will get information on potential resources of plant materials, and expertise, and also for their own R&D activities. In brief, the contents of this series have been designed to fulfill the demands of students, teachers, scientists and industry people, for small to large libraries. Students, faculties, or scientists involved in various subjects will be benefited from this series: biotechnology, bioinformatics, molecular biology, molecular genetics, plant breeding, biochemistry, ecology, environmental science, bioengineering, chemical engineering, genetic engineering, biomedical engineering, pharmaceutical science, agronomy, horticulture, forestry, entomology, pathology, nematology, and virology, just to name a few.

It has been our privilege to edit the 23 chapters of these two books, contributed by 71 scientists from 14 countries, and the list of authors includes one of the pioneers of plant transgenics Prof. Timothy C. Hall (one of the editors also); some senior scientists who have themselves edited books on plant transgenics; and many scientists who have written elegant reviews on invitation for quality books and leading journals. We believe that these two books will hopefully serve the purposes of the broad audience: those who are studying, teaching, practicing, supporting, funding, and also those who are debating for or against plant

transgenics. The first volume dedicated to "Principles and Development" elucidates the basic concepts, tools, strategies, and methodologies of genetic engineering, while the second volume "Applications and Safety" enumerates the utilization of transgenic crop plants for various purposes of agriculture, industry, ecology and environment, and also genomics research. The second volume also deliberates comprehensively on the legal and regulatory aspects; addresses the major concerns; and finally justifies the compulsion of practicing plant transgenics.

Little more detail on the contents of the volume "Transgenic Crop Plants: Principles and Development" will hopefully substantiate its usefulness. This volume focuses on the methods for constructing gene vectors, introducing these gene vectors into plant tissue, targeting gene insertion to specific tissues, methods for detecting transgene expression, generation of transgenic plants, and types of traits and bioproducts that are targeted for these technologies. The first chapter of this volume presents glimpses on these aspects and also on those related to deployment of transgenic plants.

One important factor that determines successful transgene insertion is the decision of explant type for use in transformation. A comprehensive review in Chap. 2 is provided from previous research with both herbaceous and woody plants. The use of morphogenic calli for cereals is discussed along with somewhat standardized protocols for each individual woody species. Gene transfer methods have been discussed including use of *Agrobacterium*, biolistics, electroporation, liposomes, microinjections, and bioactive beads in Chap. 3.

Once a transgene is inserted, markers have been used to either score the success of the transgene event or screen for the successful events. Although much work has been done using *npt* and *gus*, recent work has looked at marker gene removal in the final transgene product in order to belie environmental concerns. Further molecular characterization with Southern blot analysis and PCR confirm definitive transgene integration and copy number. Chapter 4 has been devoted to these critical steps.

Stable and regulated transgene expression, as described in Chap. 5, is necessary for the transgenic plant to express the trait of interest for further research or commercial applications. The use of constitutive and tissue-enhanced promoters along with attention to attachment regions within the DNA, introns, RNA integrity factors, and transcription factors will determine transgene expression and the levels thereof. Besides using transgenes for introduction of nucleic acid for novel trait production, transgenes have also been used to silence native genes for applications such as resistance to nematodes, insects, and viruses. As interesting are the applications to reduce production of compounds in some plants, such as caffeine in coffee and sulfur metabolites in onions, that are disagreeable to portions of the human populations. All of these silencing events rely on RNAi technology to degrade native RNA for those traits of interest. Chapter 6 provides a commentary on the employment of RNAi technology and the implications and outcome of expression and gene silencing have been explicated in Chap. 7.

Researchers have also been successful with organelle transfer, which has applications to molecular pharming, as have been enumerated in Chap. 8. This technique can be employed to overcome some transgene expression difficulties. Cell culture

biosynthesis and metabolic engineering are the focus of the last two chapters (Chaps. 9 and 10) in this volume and these chapters offer an intriguing look at research into production of high-value energy and medicinal products, secondary metabolites, and plants with attractive esthetic qualities. Because we still have a rudimentary understanding of many biochemical pathways, we are continuing to gain new knowledge and insight into pathway function, but commercial plant systems are still lacking in most desirable traits when economic viability, environmental safety, and sustainability are taken into account.

We thank the 31 scientists from 9 countries for their elegant and lucid contributions to this volume and also for their sustained support through revising, updating, and fine-tuning their chapters. We also acknowledge the recent statistics we have accessed from the web sites of Monsanto Company on "Conversations about Plant Biotechnology" and "International Service for the Acquisition of Agri-Biotech Applications on ISAAA Brief 39-2008: Executive Summary" and used them in this preface and elsewhere in the volume.

We enjoyed a lot our Clemson-Purdue-Texas A&M triangular interaction, constant consultations, and dialogs while editing this book and also working with the editorial staff of Springer, particularly Dr. Sabine Schwarz, who had been supportive since the inception till the publication of this book.

We look forward to suggestions from all corners for the future improvement of the content and approach of this book volume.

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Abbreviations

2, 4-D	2, 4-Dichlorophenoxyacetic acid
2-DOG	2-Deoxyglucose
4MI	4-Methylindole
4-MT	4-Methyltryptophan
4-MUGIuc	4-Methylumbelliferyl glucuronide
5MT	5-Methyltrypthopan
7MT	7-Methyl-DL-tryptophan
AA	Arachidonic acid
aadA/addA	Aminoglycoside 3'-adenyl transferase
ABC	*Arabidopsis* ATP-binding cassette
ABW	Aluminium borate whiskers
ACC	Aminoglycoside acetyltransferase
ADC	Arginine decarboxylase
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
AEC	S-Aminoethyl l-cysteine
AG	*Arabidopsis* agamous
AHAS	Acetohydroxyacid synthase
AK	Aspartate kinase
ALA	Aminolaevulinic acid
ALA	α-Linolenic acid
AL-PCR	Adaptor ligation-PCR
ALS	Acetolactate synthase
amiRNA	Artificial micro-RNA
AMV	Alfalfa mosaic virus
ANS	Anthocyanidin synthase
AP2	*APETALA2* gene
APH	Aminoglycoside phosphotransferase
*aro*A (*epsps*)	5-Enolpyruvylshikimate-3-phosphate synthase gene
ARS	Autonomously replicating sequence
*ars*C	Arsenic reductase gene

AS	Amyrin synthase
AS	Anthranilate synthase
ATP	Adenosine tri-phosphate
AtTSB1	*Arabidopsis* tryptophan synthase beta 1 gene
BA	Benzylaminopurine
BADH	Betaine aldehyde dehydrogenase
bar (*pat*)	Phosphinothricin acetyltransferase gene
BMS	Black mexican sweet
Bnx	Bromoxynil nitrilase gene
BOAA	β-*N*-Oxalylamino-L-alanine
BSMV	Barley stripe mosaic virus
BSV	Banana streak virus
Bt	*Bacillus thuringiensis*
BYDV	Barley yellow dwarf virus
C4H	Cinnamate 4-hydroxylase
Cah	Cyanamide hydratase
CaMV	Cauliflower mosaic virus
CAS	Cycloaretenol synthase
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary-DNA
CDPK	Calcium dependent protein kinase
CHI	Chalcone isomerase
CHR	Chalcone reductase
CHS	Chalcone synthase
CKI1	Cytokinin-Independent 1
CMV	Cytomegalovirus
cp4	Glyphosate resistant 5-enolpyruvylshikimate-3-phosphate synthase
*cpt*II	Carnitine palmitoyltransferase II gene
CRC	Chimeric transcription factor
CRY	Cryptochrome
CSIRO	Commonwealth Scientific and Industrial Research Organization
CsVMV	Cassava vein mosaic virus
CUP1	Yeast metallothionein gene
CYP	Cytochrome P450
CYP	Cytochrome P450 gene
DAAO	D-Amino acid oxidase
DEF	Peptide deformylase
DFR	Dihydroflavonol reductase
DHA	Docosahexaenoic acid
DHDPS	Dihydrodipicolinate synthase
DHFR	Dihydrofolate reductase
DHK	Dihydrokaempferol

DHM	Dihydromyricetin
DHPS	Dihydrodipicolinate synthase
DHPS	Dihydropteroate synthase
DHQ	Dihydroquercetin
DIG	Digoxigenin
DQR	Dihydroquercerin-4 reductase
dsRNA	Double stranded-RNA
EBV	Epstein barr virus
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentanoic acid
EPSP	5-Enolpyruvylshikimate-3-phosphate
EPSPS	5-Enol-pyruvyl shikimate-3-phosphate synthase
EREBP	Ethylene response element binding protein
ERF	Ethylene response factor
ESR1	Enhancer of shoot regeneration 1
F3′5′H	Flavonoid 3′, 5′-hydroxylase
F3′H	Flavonoid 3′-hydroxylase
F3H	Flavanone 3b-hydroxylase
FAD3	Fatty acid desaturase 3
FAO	Food and Agricultural Organization
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FLC	Flowering locus C gene
FMV	Figwort mosaic virus
FNSII	Flavone synthase II
galT	UDP-glucose:galactose-1-phosphate uridyltransferase
GBSS	Granule bound starch synthase gene
GFP	Green fluorescent protein
gfp	Green fluorescent protein gene
GM	Genetically modified
GOI	Gene of interest
GOX	Glyphosate oxidase
gox	Glyphosate oxidoreductase gene
GS	Glutamine synthase
GSA-AT	Glutamate-1-semialdehyde aminotransferase
gshI	Glutathione synthase I gene
GST	Glutathione-S-transferase
gus	β-Glucuronidase gene
GUS	β-Glucuronidase
H6H	Hyoscyamine-6-hydroxylase
HAS	Human serum albumin
HAT	Histoneacetyl transferase
HD	Histone deacetylase
hph (*hpt, aph*IV)	Hygromycin phosphotransferase gene

hpRNA	Hairpin RNA
HPT	Hygromycin phosphotransferase
HSP	Heat shock protein
IAA	Indole acetic Acid
IFS	Isoflavone synthase
IM	Imidazolinone
IME	Intron-mediated enhancement
IPT	Isopentyl transferase
KN1	Homeobox gene knotted1
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acid
LDC	Lysine decarboxylase
LF	Lachrymatory factor
LFS	Lachrymatory factor synthase
LRE	Light responsive elements
LT	Lysine and threonine
Luc/*lux*	Luciferase gene
LUS	Lupeol synthase
MAR	Matrix associated/attachment region
MCA	Metabolic control analysis
mer	Mercuric ion reductase gene
MMV	Mirabilis mosaic virus
mRNA	Messenger-RNA
MT1	Metallothionein 1 gene
MT2	Metallothionein 2 gene
Mtx	Methotrexate
NAA	Naphthalenacetic acid
NoGA2ox3	Oleander gibberellic acid 2-oxidase gene 3
NOS	Nopaline synthase
nos	Nopaline synthase gene
nptII	Neomycin phosphotransferase II
*npt*II	Neomycin phosphotransferase II gene
OCS	Octopine synthase
ODC	Ornithine decarboxylase
OMT	L-O-Methylthreonine
OPH	Organophosphate hydrolase
ORF	Open reading frame
OxO	Oxalate oxidase
PAL	Phenylalanine ammonia lyase
PAT	Phosphinothricin acetyltransferase
PCR	Polymerase chain reaction
PDR	Pathogen-derived resistance
PEG	polyethylene glycol
Pflp	Pepper ferredoxin-like protein

PGA	Plant growth activator
PGL34	Plastoglobulin 34
PGT	p-Hydroxybenzoate geranyltransferase
PHB	p-Hydroxybenzoate/Polyhydroxybutyrate
PHY	Phytochrome
PIG	particle in-flow gun
PMI	Phosphomannose isomerase
pmi	Phosphomannose isomerase gene
PPO	Protoporphyrinogen oxidase
PPT	Phosphinothricin
ppt	Phosphinothricin acetyltransferase gene
PTGS	Post-transcriptional gene silencing
PUFA	Polyunsaturated fatty acids
PVX	Potato virus X
PVY	Potato virus Y
RNAi	RNA interference
ROL/*rol*	Root locus
RT-PCR	Reverse transcription-PCR
SAAT	Sonication-assisted *Agrobacterium*-mediated transformation
SAM	S-adenosylmethionine
SAUR	Small auxin up RNA
ScBV	Sugarcane bacilliform badnavirus
SDA	Stearidonic acid
siRNA	Short interfering RNA
SOP	Standard operating procedures
SPT	Streptomycin phosphotransferase
STK	Seedstick
SU	Sulfonylureas
SV40	Simian virus 40
TAIL-PCR	Thermal asymmetric interlaced-PCR
TD	Threonine deaminase
TDC	Tryptophan decarboxylase
T-DNA	Transferred DNA
Ti plasmid	Tumour-inducing plasmid
TILLING	Target induced local lesions in genomes
TMV	Tobacco mosaic virus
TP	Triazolopyrimidine
TSP	Total soluble protein
TSSR	Tuber-specific and sucrose responsive
T-strand	Transferred strand
UDP	Uridine diphosphate
UFGT	UDP-glucose:flavonoid 3-O- glucosyltransferase
uidA (*gusA*)	β-glucuronidase gene
USDA	United States Department of Agriculture

UTR	Untranslated region
UV	Ultraviolet
VIGS	Virus induced gene silencing
WIN1	Wax inducer 1
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D- glucuronide
YAC	Yeast artificial chromosome
ZAT1/*ZntA*	Zinc transporter gene

Chapter 1
Generation and Deployment of Transgenic Crop Plants: An Overview

Michael R. Davey, Jaya R. Soneji, M. Nageswara Rao, Sofia Kourmpetli, Anjanabha Bhattacharya and Chittaranjan Kole

1.1 Introduction

As biotechnology increasingly affects almost all aspects of human life, it is essential that the science behind this technology is explained in simple terms to the public to eliminate the misconceptions that may inhibit its acceptability. The basic question that is often asked is what is a gene, a promoter and a terminator? Genes are the basic units of heredity, composed of DNA sequences, which are transmitted from parents to offspring and which, independently or in combination with other genes, control specific traits in an organism. These traits may be, for example, plant height, flower color, fruit and seed size together with regulatory processes, such as assimilate partitioning and drought resistance. Genes are the basis for both the similarity and differences that exist among organisms, and are transmitted from one generation to another. Promoters are DNA sequences that are recognized by RNA polymerase in plant cells and that initiate and regulate transcription, the initial and most important step of gene expression. Terminators are those sequences that command or signal the termination of transcription.

It is possible to identify and to isolate genes from plants, animals, and microorganisms, to modify their promoters, structural sequences and terminators, and to introduce and express chimeric genes in the same or other genus, species, or cultivar. Consequently, it is feasible to control or modify physiological processes. Gene manipulation, combined with the ability to induce cultured plant cells to express their totipotency leading to the regeneration of fertile plants, provides a

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unique opportunity to extend the genetic pool available to breeders for crop improvement.

The successful development of transgenic plants necessitates a reliable tissue culture regeneration system, gene construct(s), suitable vector(s) for transformation and efficient procedures to introduce desired genes into target plants. Once transformation has been performed, it is essential to recover and to multiply the transgenic plants. The latter must be characterized at the molecular and genetic levels for stable and efficient gene expression (Sharma et al. 2005). It may also be necessary to transfer the introduced genes to elite cultivars by conventional breeding.

Prime targets for genetic manipulation include modification of plants to enhance their tolerance to the herbicides used to control weeds, and to confer resistance to insects, bacteria, fungi, and viruses, since these agents account for major crop losses. Other targets include the genetic engineering of plants for biosynthesis of health-care products, increased nutritional value, extension of the shelf-life of crops that deteriorate rapidly following harvest, and tolerance to abiotic stress. Similarly, although not essential for human existence, modification of the esthetic appeal of plants has considerable commercial potential.

1.2 Target Cells and Organelles for Genetic Transformation

A reliable tissue culture-based shoot regeneration system is a pre-requisite for plant genetic transformation. The recognition that, under optimum hormonal and nutritional conditions, somatic cells are totipotent and can be stimulated to develop into whole plants in vitro via organogenesis (shoot formation) or somatic embryogenesis, forms the basis of regeneration in tissue culture (Sharma et al. 2005). Genetic transformation without plant regeneration is of limited or no value. Hence, the identification of explants (cells/tissues/organs) that are capable of regenerating into plants is fundamental to any transformation procedure. Isolated protoplasts (Davey et al. 2005), callus and suspension cultured cells (Rachmawati and Anzai 2006), thin cell layers (Soneji et al. 2007a), leaf disks (Li et al. 2007), root sections (Huang and Ma 1992), stem segments (Song et al. 2006), floral tissues (Zale et al. 2008), epicotyls (Soneji et al. 2007b), hypocotyls (Wang and Xu 2008), cotelydonary nodes (Yi and Yu 2006), and axillary buds (Manickavasagam et al. 2004) have been used for genetic transformation. Explants of mature organs have also been used as target material in transformation experiments to overcome juvenility (Cervera et al. 1998). Tissue culture systems for several plants have been summarized (Khachatourians et al. 2002; Curtis 2004; Loyola-Vargas and Vázuez-Flota 2005) together with aspects of gene introduction into target plants using such systems (Birch 1997; Newell 2000; Sharma et al. 2005; Davey et al. 2008).

Shoot regeneration from cultured cells may lead to chromosomal or genetic variation known as “somaclonal variation.” This variation may be useful or detrimental. Tissue culture also requires extensive facilities for maintenance and manipulation of axenic explants, which is labor intensive and expensive.

Consequently, approaches have been reported that reduce or eliminate in vitro procedures. For example, genes have been inserted into pollen and the latter used for fertilization to produce transgenic seed (Saunders and Matthews 1995; Häggman et al. 1997), while Clough and Bent (1998) described a "floral dip" procedure that is discussed later.

In most investigations, gene insertion has been directed primarily to the nuclei of recipient plant cells. Additionally, plastid transformation has been established in several laboratories (Heifetz 2000; Daniell et al. 2002; Maliga 2002, 2004). Extension of plastid transformation to more species constitutes a logical step in the development of genetic manipulation technology (Bock and Khan 2004) as plastid transformation has several advantages for the engineering of gene expression in plants. These advantages include 10–50 times greater transgene expression in plastid genomes, compared to nuclear-inserted genes (Liu et al. 2008a). The plastid genome provides readily obtainable high protein concentrations and the possibility of expressing multiple proteins from polycistronic mRNAs from a single promoter (Maliga 2002). Importantly, uniparental plastid gene inheritance in most crop plants prevents pollen transmission of foreign DNA (Heifetz 2000). As transgenes integrate into the plastid genome via homologous recombination, this facilitates targeted gene replacement and precise transgene control, while sequestration of foreign proteins in plastids prevents adverse interactions with the cytoplasmic environment. Maliga (2004) and Verma and Daniell (2007) discussed the design of vectors for plastid transformation and the selection of transplastomic plants. To date, plastid transformation has been reported in cabbage, lettuce, oilseed rape, petunia, poplar, potato, tobacco, and tomato, with transplastomic plants being regenerated by organogenesis in these cases, or by somatic embryogenesis in carrot, cotton, rice, and soybean (Verma et al. 2008). Extension of plastid transformation to other major crop plants still necessitates reproducible explant, cell, or protoplast-to-plant regeneration systems.

1.3 Methods for Introducing Genes into Plants

Transformation of plants involves the stable introduction of DNA sequences usually into the nuclear genome of cells capable of developing into a whole transgenic plant (Sharma et al. 2005). Once a reliable shoot regeneration system is available, foreign DNA can be introduced into cells by either vector-mediated or direct transfer. Although the technology associated with the construction of chimeric genes is becoming more routine and simple, the transformation process itself remains a comparatively rare event. Consequently, the procedure must be robust and combine reproducible culture of recipient plant cells with efficient gene delivery. Gene transfer experiments focus mainly on maximizing the efficiency of recovery of stably transformed plants, and extending the range of species that can be engineered using a specific procedure.

Agrobacterium-mediated gene transfer and direct DNA transfer into cells by microprojectile bombardment (Fig. 1.1) are the most widely exploited methods for introducing genes into plants because of their ability to transform intact, regenerable tissues and organs. Although aspects of the precise molecular events of *Agrobacterium*-mediated gene delivery are still not fully understood, particularly the transfer and integration of the T-DNA (transferred DNA) from the bacterial tumor-inducing (Ti) plasmid of *Agrobacterium* into the nuclei of recipient plants, *Agrobacterium*-mediated gene delivery remains the preferred method of plant transformation in many laboratories. Lacroix et al. (2006a, 2006b) and Tzfira and Citovsky (2006) proposed mechanisms for the process. Knowledge of foreign gene integration into plant genomes is essential for precise gene targeting in the future.

Immersion of totipotent explants in a suspension of *Agrobacterium* is the main procedure for plant transformation. Several parameters affect transformation, including bacterial virulence, incubation temperature, age of the bacterial suspension, and the cocultivation period of the bacteria with the explants (Gelvin 2003; Wu et al. 2003). Sonication promotes gene delivery (Liu et al. 2006). In planta procedures have been developed to simplify the transformation procedure. Thus, the "floral dip" technique (Clough and Bent 1998) involves immersion of developing flowers in suspensions of *Agrobacterium*, followed by growth of the plants to maturity, the harvesting and germination of seeds, and the selection of transformed seedlings. This procedure, used routinely to transform *Arabidopsis thaliana*, has facilitated progress in understanding the genetics of this plant that is exploited extensively as a model in plant genetics and molecular biology. Chung et al. (2000) compared floral spraying with the floral dip procedure and reported comparable results with the two methods, enabling floral spraying to be used for transforming plants which are too large for the floral dip approach. Probably, in planta techniques will assume increasing importance for gene delivery.

Particle (microprojectile) bombardment has also been exploited extensively for plant transformation (Sharma et al. 2005; Davey et al. 2008) with instruments such as the helium driven HE-1000 device, facilitating technology transfer between laboratories. Microprojectile systems involve high-velocity particles penetrating cell walls and introducing DNA into cells, circumventing the host range limitations of *Agrobacterium*. This transformation procedure is versatile, independent of plant cell type and genotype, and has permitted the transformation of some of the most recalcitrant plants, such as cereals and legumes (Altpeter et al. 2005). Importantly, simple gene constructs, comprising only a promoter, the gene coding sequence and a terminator, may be used for transformation. A criticism of particle bombardment and *Agrobacterium*-mediated gene delivery is the complexity of patterns often associated with the integration of genes into recipient plants, especially with particle delivery. This necessitates detailed molecular analyses to select individuals carrying simple integration events, as such transformed plants are more applicable to longer-term breeding programs. Undoubtedly, the two procedures will continue

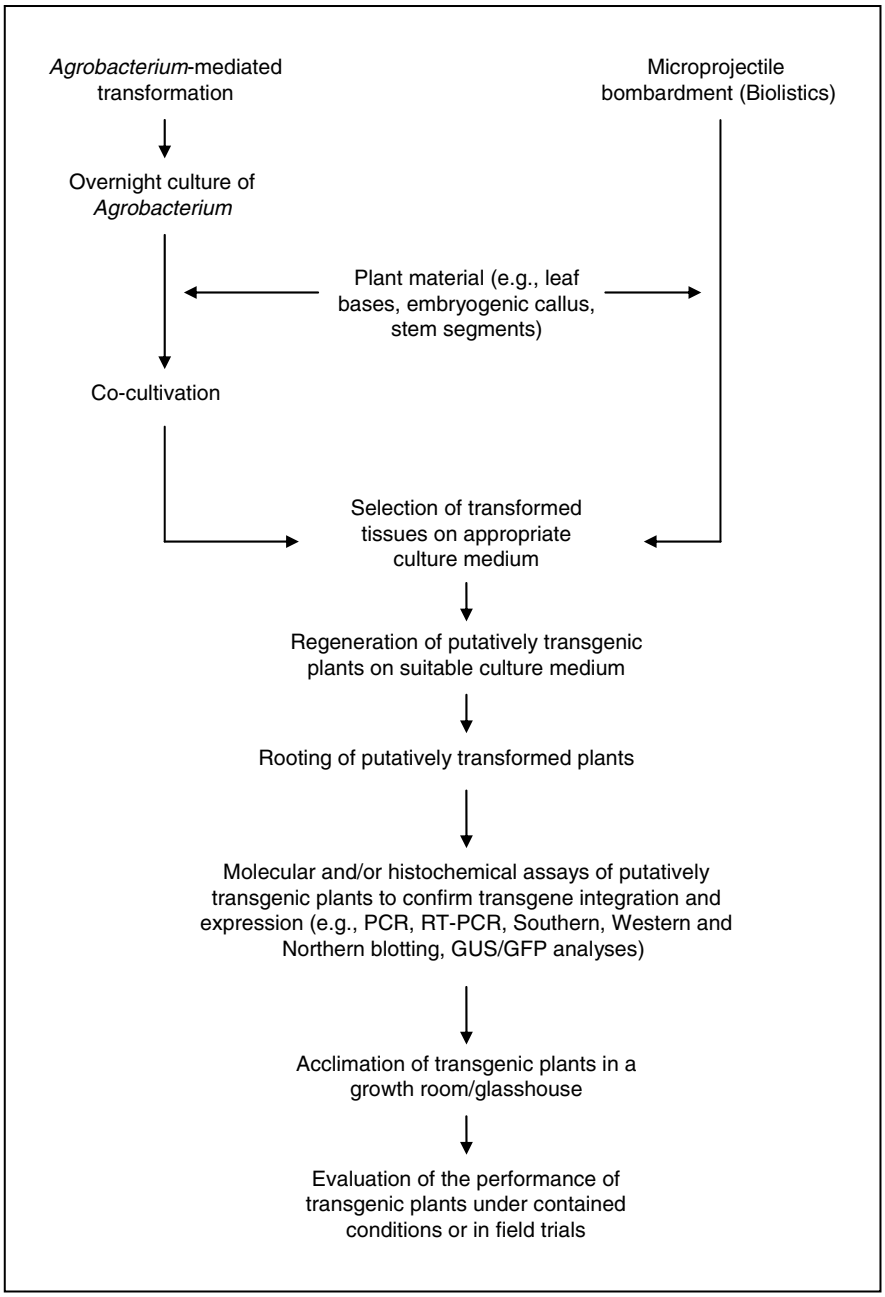

Fig. 1.1 A generalized flow chart depicting the steps involved in plant transformation

to be exploited routinely for gene delivery to plants, the procedure used depending upon the product required and the expertise of the personnel.

Other gene delivery procedures have been exploited, including uptake of DNA into isolated protoplasts, treatment with polyethylene glycol, and/or electroporation inducing DNA uptake. However, the development of robust protoplast-to-plant systems is a labor-intensive, specialized part of the procedure. Several parameters influence transformation, including the stage of the cell cycle of the recipient protoplasts, temperature, pH, and the intensity and duration of the electric field. Even with optimal conditions, the frequency of stable transformation is low and rarely exceeds one transformed cell in every 10^4 treated protoplasts. Protoplast transformation was the procedure of choice for monocotyledons, particularly cereals such as rice, but was superseded by particle bombardment and, more recently, by *Agrobacterium*-mediated gene delivery. Rakoczy-Trojanowska (2002) and Sharma et al. (2005) discussed transformation procedures involving micro- and macroinjection, the use of silicon carbon fibers, and pollen-tube-mediated DNA delivery. Virus-based DNA delivery methods have been reported (Chung et al. 2006). The real success and application of several transformation procedures remains unclear. Specific crops necessitate particular adaptation of techniques to generate transgenic plants, an excellent example being provided by some of the difficulties encountered in applying *Agrobacterium*-mediated gene delivery developed for rice to other cereals (Shrawat and Lörz 2006). However, gene sequencing, as in rice (Matsumoto et al. 2005), and general advances in plant bioinformatics, will facilitate broader application of transformation technology.

1.4 Vector Construction and Genes for Plant Transformation

Vector development has proceeded from the cointegration of foreign genes into the T-DNA region of Ti plasmids, to the construction of disarmed binary and superbinary vectors (Komori et al. 2007; Davey et al. 2008). As Tzfira et al. (2007) explained, although binary vectors were initially revolutionary, subsequent generations of vectors have had more versatility, often being designed for specific transformation purposes (Chung et al. 2005). Some vectors have incorporated recombinase-mediated gene cloning (Karimi et al. 2002). Importantly, advances in vector construction have enabled *Agrobacterium*-mediated transformation to be exploited for gene introduction into monocotyledons (Cheng et al. 2004), as well as dicotyledons. New gene expression technologies developed for nonplant systems rapidly become adapted and exploited in plant biology (Tzfira et al. 2007). This emphasizes the necessity for plant biologists to recognize and exploit developments in fields of research other than their own. A schematic representation of the steps involved in the construction of vectors for plant transformation is shown in Fig. 1.2.

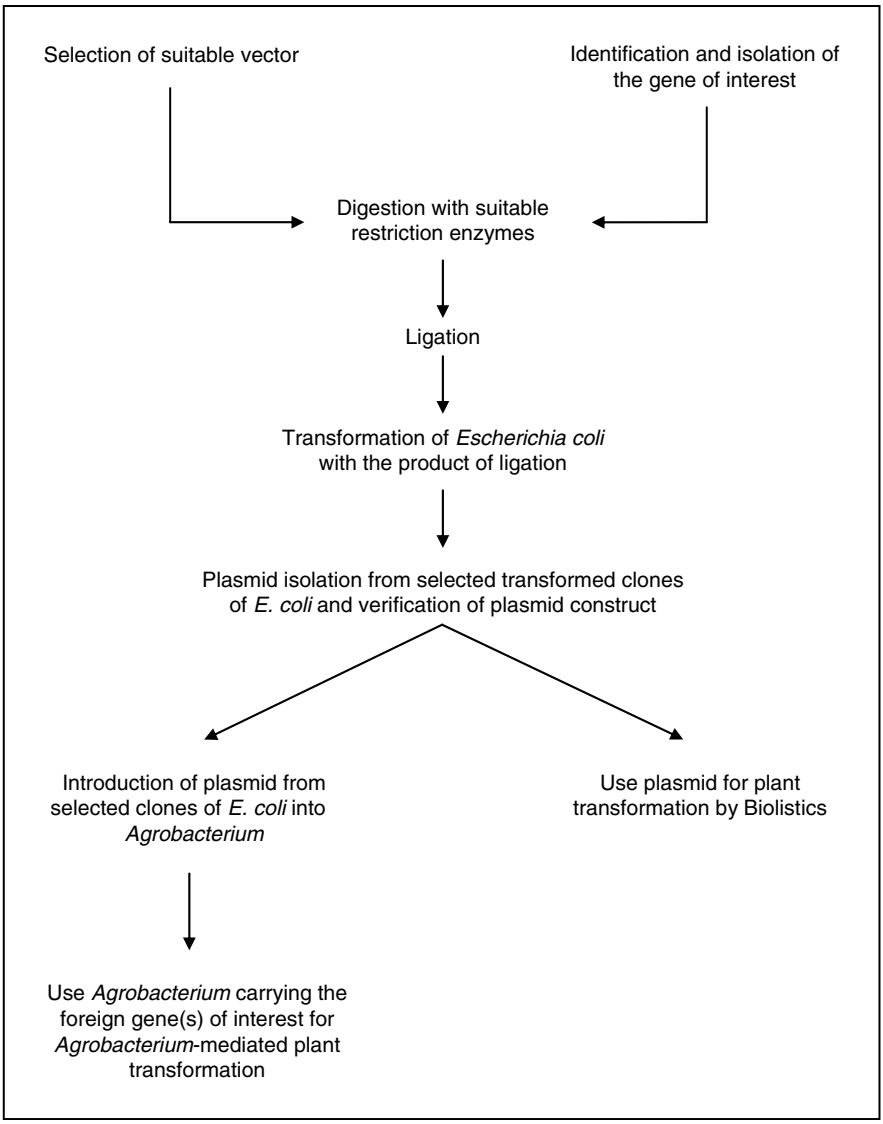

Fig. 1.2 Flow chart depicting the steps involved in the construction of vectors for plant transformation

1.4.1 Promoters for Plant Transformation

Efficient and reliable procedures are essential for constructing vector(s) for plant genetic engineering. Venter (2007) highlighted the importance of focusing attention on promoter construction, because the choice of promoter and its fine-tuning

determine constitutive, spatial, and/or temporal transgene expression. Considerable effort has focused on gene promoters. Efficient expression of genes is assured only when they are controlled by plant-derived promoters, or by promoters that are active in plant cells, such as the cauliflower mosaic virus 35S promoter (CaMV 35S). In early transformation assessments, the choice of promoter was governed by promoter availability. The *nos* promoter from the nopaline synthase gene of the T-DNA of the Ti plasmid of *A. tumefaciens* was one of the first to be used in plant genetic engineering, with the 35S promoter from CaMV also featuring in many of the early transformation assessments. Subsequently, other constitutively expressed viral promoters were evaluated, including those from cassava vein mosaic virus (CsVMV), sugarcane bacilliform badnavirus (ScBV), and figwort mosaic virus (Samac et al. 2004; Govindarajulu et al. 2008). The CaMV 35S promoter may have a negative effect on transgene expression in some plants (Yoo et al. 2005). A limitation of the promoters of viral origin is that host plants may recognize and inactivate these sequences (Potenza et al. 2004). However, this may be negated by using promoters of plant origin. Indeed, several promoters including those from *Medicago truncatula* (Xiao et al. 2005), *Vigna radiata* (Cazzonelli et al. 2005), and the tobacco EI1α together with the Cab promoters (Aida et al. 2005) have been evaluated.

Constitutive expression at the incorrect time may have a serious negative effect on plant development, emphasizing the need to refine the promoters for transgene expression. Tissue-specific promoters fulfill this requirement. Examples include a tissue-specific promoter driving a β-1, 3 gluconase gene in pea (Buchner et al. 2002), promoters from fruit-ripening and seed-specific genes (Zakharov et al. 2004) particularly seed storage glutelin genes (Qu et al. 2008) and promoters of glycoproteins in tubers and roots. Flower-specific promoters have application in the genetic manipulation of fruit trees and ornamental plants (Annadana et al. 2002; Sassa et al. 2002). Comparisons of promoter function are important, a cotton α-globulin promoter being evaluated in cotton, *Arabidopsis*, and tobacco (Sunilkumar et al. 2002). Potenza et al. (2004) provided a schematic representation of the sources of many promoters. Tissue-specific promoters have been combined with RNA interference (RNAi) technology to modify flower pigmentation (Nakatsuka et al. 2007a). Modification of promoters may result in changes in tissue and developmental specificities (Kluth et al. 2002). Promoters of considerable potential are those associated with the interaction of plants and microorganisms, such as root-specific promoters involved in nutrient uptake and legume-*Rhizobium* symbiotic associations. These promoters from green tissues confer light-inducible and tissue-specific expression. Cell-type-specific promoters are available, such as those from trichomes, guard cells and stomata, root hairs, phloem (Zhao et al. 2004; Guan and Zhou 2006), and cortical cells (Fruhling et al. 2000). Vectors for plastid transformation normally employ promoters from the plastid genomes of the target plants.

Some plant promoters are induced by biotic and abiotic stress (Pino et al. 2007), wounding (Yevtushenko et al. 2004; Luo et al. 2006), iron deficiency (Kobayashi et al. 2007), and exogenously applied chemicals. The latter include antibiotics,

steroids, copper, ethanol (Peebles et al. 2007), inducers of pathogen-related proteins, herbicide safeners and insecticides (Padidam 2003). Synthetic promoters have been assembled, such as a chimeric endosperm-specific promoter for cereal transformation (Oszvald et al. 2008). Liu et al. (2008b) constructed a novel pollen-stigma and carpel-specific promoter, which has potential in controlling pollen and seed-mediated gene flow from genetically manipulated plants. However, some synthetic promoters are unsuitable for plant transformation. For example, the (AocS)(3)AmasPmas promoter driving the *bar* gene for herbicide tolerance inhibited shoot regeneration (Song et al. 2008). Synthetic promoters, with the minimum of sequence similarity, could reduce homology-dependent gene silencing in transgenic plants during gene pyramiding experiments. Indeed, the availability of a broad spectrum of promoters that differ in their ability to regulate temporal and spatial expression patterns of transgenes could increase dramatically the success of transgenic technology (Potenza et al. 2004). Promoter development is still in its infancy. Major advances in transcriptomics, proteomics, and genome sequencing (Yu et al. 2007) will contribute to future development of promoters to drive gene expression in specific cells and tissues.

The correct assembly of constructs for plant transformation is fundamental for maximum gene expression at the correct time in target tissues (Butaye et al. 2005). The merit of bidirectional as well as unidirectional promoters necessitates consideration. Undoubtedly, continued advances in plant genetics, bioinformatics, systems biology, and high through-put gene expression technology will be crucial in predicting coordinated gene expression and the design of synthetic promoters. Terminator sequences must also originate from plant sources or from plant pests such as the CaMV or *Agrobacterium*. Although most investigations are targeted to maximizing gene expression in transgenic plants, the ability to silence genes is equally important in some cases, virus-induced gene silencing (VIGS) being a way of down-regulating expression (Robertson 2004).

1.4.2 Reporter and Selectable Marker Genes

Transformation, being a rare event, requires an efficient selection system to distinguish between transformed and nontransformed plant cells. Reporter genes enable cells and tissues to be monitored soon after the transformation procedure to assess the success of a specific construct and/or protocol. Such genes may permit the manual or automated selection of transformed from nontransformed cells, but do not enable transformed cells to outgrow their nontransformed counterparts in culture. In contrast, selectable marker genes provide transformed cells with a competitive advantage, enabling them to outgrow nontransformed cells in vitro, usually in the presence of specific substrates in the culture medium.

Although more than 50 genes have been exploited in nuclear and plastid transformation strategies, only a limited number are used routinely (Miki and McHugh 2004). The *uidA* (*gusA*) gene for β-glucuronidase is a versatile reporter.

In fluorometric and histochemical assays, cleavage of the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) by β-glucuronidase results in an indigo compound that is readily observed in transformed cells. A disadvantage of the GUS assay is its destructive nature. Consequently, it has been superseded in many investigations by more versatile, nondestructive assays based on expression of the luciferase (*luc*) gene, or the green fluorescent protein (*gfp*) gene, the latter from the jellyfish, *Aequorea victoria*. Mutant versions of the *gfp* gene that emit blue, cyan, and yellow light are available. Novel proteins from reef coral organisms that fluoresce cyan, red, green, and yellow have also been developed as nondestructive reporters for plant transformation (Wenck et al. 2003). Importantly, significant differences in the excitation and emission wavelengths of some of these proteins permit simultaneous visualization of more than one of these fluorescent proteins in transformed cells. Dixit et al. (2006) emphasized the importance of fluorescent proteins to image dynamic processes within plant cells, highlighting some of the practical issues in exploiting these proteins for live cell imaging. Genes for anthocyanin and carotenoid biosynthesis have also been used to visualize transformed cells prior to their manual selection.

Selection systems have been reported that encourage the growth of transformed cells, although "escapes" may occur, with some nontransformed cells growing in the presence of a selective agent. Commonly used selection systems employ tolerance to antibiotics, particularly kanamycin, encoded by the neomycin phosphotransferase (*npt*II) gene, and to hygromycin through expression of hygromycin phosphotransferase (*hph*, *hpt*, *aph*IV) genes. Phosphinothricin and glyphosate have featured in selection systems based on herbicide resistance, tolerance to phosphothricin being encoded by expression of the *bar* (*pat*) gene, while the *aroA*, *cp4*, and *epsps* and *gox* genes confer tolerance to glyphosate. Streptomycin and spectinomycin have been used to select transplastomic plants. Recently, Pinkerton et al. (2008) introduced resistance based on the enzyme organophosphate hydrogenase, encoded by the bacterial *opd* gene, to generate a new scorable and selectable marker system for transgenic plants. Some investigators have focused on plant genes as selectable markers. For example, Yemets et al. (2008) based selection on a modified plant α-tubulin gene that conferred resistance to dinitroaniline herbicides, with trifluralin as the selective agent. Ogawa et al. (2008) used a mutated rice acetolactate synthase gene to select transgenic plants of wheat. Acetolactate synthase catalyzes the first step in the biosynthesis of the essential branched-chain amino-acids, isoleucine, leucine, and valine, and is a target enzyme for several herbicides. Other procedures have incorporated toxic drugs and metabolite analogs into the culture medium. Genes that stimulate cytokinin biosynthesis stimulated shoot regeneration from transformed cells without the need for selection based on toxic compounds (Zuo et al. 2002). The *Escherichia coli pmi* gene for phosphomannose isomerase converts mannose-6-phosphate, an inhibitor of glycolysis, to fructose-6-phosphate, an intermediate in glycolysis. Expression of *pmi* in plant cells allows transformed cells to grow on medium containing mannose, as in the case of transgenic flax, following *Agrobacterium*-mediated transformation (Lamblin et al. 2007). Future legislation will, almost certainly, demand the elimination of antibiotic resistance genes as

selectable markers. Although selectable markers are generally indispensable in plant transformation protocols, they are not required once transgenic plants have been generated. General strategies to eliminate selectable marker genes have been reported. Jia et al. (2006) exploited the Cre/*lox* site-specific recombination system, while Charng et al. (2008) developed an inducible transposon system to terminate selectable marker gene function in transgenic plants. More detailed description of selection systems is presented by Miki and McHugh (2004) and Davey et al. (2008).

1.5 Methods for Screening of Genes Introduced into Putatively Transformed Plants

The strategies used for screening transformed plants usually depend on the type of selectable marker and/or reporter gene used. When an antibiotic resistance gene is employed as a selectable marker, screening is performed by culturing the transformed cells on a medium containing that particular antibiotic (Soneji et al., 2007b, 2007a). In the case of reporter genes, screening is for the distinctive phenotype (Chalfie et al. 1994). However, putative transgenic plants selected by scoring for the presence of selectable marker and/or reporter genes need to be evaluated for the integration and expression of the transgene(s) to minimize escapes. Polymerase chain reaction (PCR)-based screening techniques are used to assess the presence of a specific DNA sequence of the foreign gene of interest, or the selectable marker/reporter gene by screening putative transgenic plants with primers specific to these gene(s) (Xu et al. 2005; Soneji et al. 2007b, 2007a). Southern hybridization confirms the presence of transgenes and their copy number (Bhat and Srinivasan 2002). Enzyme-linked immunosorbent assay (ELISA) is the preferred method to detect the presence of a specific protein produced by a transgene in a recipient plant. Real-time polymerase chain reaction (RT-PCR) is utilized when more than one gene needs to be analyzed by PCR, along with the detection of the copy number of the desired gene(s) (Yuan et al. 2007).

1.6 Gene Expression in Transgenic Plants

Integration of transgenes into the genomes of plants is a random process, necessitating investigations of their expression in transformed plants. Expression is influenced by several parameters, including the site and pattern of integration, the location of heterochromatic regions, the presence of enhancer elements, the nature of the promoter, gene copy number, truncation, rearrangement, silencing and the presence of any DNA sequences from the vector into which the foreign DNA has been cloned. Although some of these factors can be circumvented by experimental design, it is still necessary to correlate phenotypic differences between transgenic

and control plants with transgene expression (Page and Minocha 2005). Thus, transgenic plants require detailed phenotypic, physiological, and molecular analyses to complete their characterization. Techniques such as Western blotting, Northern blotting, ELISA, and quantification and localization of mRNA transcripts are used to analyze transgene expression. These assessments are essential, especially when transgenic material is incorporated into breeding programs.

The use of genetic manipulation in crop improvement also requires transgenes to be expressed either constitutively or in specific cell or tissue types, often at definite stages of plant development (Perret et al. 2003). Although individual transgenic plants within a population may be phenotypically identical, generally they all differ in some subtle way at the molecular level. This emphasizes the requirement to generate as many transgenic plants as possible from an individual experiment and to analyze the maximum number of the regenerants at the phenotypic and molecular levels (Bhat and Srinivasan 2002). Currently, there is no reliable procedure to target foreign genes to specific regions of the genome of plants. It may also be necessary to test individual promoters to establish their expression patterns in different species (Perret et al. 2003). While gene targeting by homologous recombination is potentially extremely important, the development of a routine procedure that incorporates this process remains a major challenge (Cotsaftis and Guiderdoni 2005).

In order to determine the value and application of transformed plants, it is important to understand the inheritance and stability of introduced gene(s). Transmission and segregation analyses of the transgene(s) in subsequent progenies allow insight into transgene inheritance (Yin et al. 2004). *Agrobacterium*-mediated transformation, as well as direct DNA uptake, enables foreign genes to be integrated at a single Mendelian locus, regardless of copy number (Spencer et al. 1992). Stably integrated transgenes are usually inherited in a dominant, Mendelian fashion. However, in subsequent generations, some instability may be observed probably due to rearrangements or methylation of the T-DNA region, and/or to homologous recombination between copies of the transgene inserted into the same nucleus. A non-Mendelian segregation pattern is usually associated with unstable transformation or poor transgene expression (Limanton-Grevet and Jullien 2001).

1.7 Target Genes for Genetic Transformation

Major advances in gene isolation, vector construction, and DNA delivery enable plants to be modified for specific traits, providing an important underpin to conventional breeding. Although genetic engineering reduces the time to integrate desired genes into target plants, it will not replace gene manipulation by sexual hybridization. It has been emphasized that many of the constraints associated with conventional breeding can be overcome by advances at the molecular level (Dalal et al. 2006). Transgenes to be introduced into plants are selected on the basis of their economic/agronomic importance. Recent advances in DNA array technology allow

researchers to detect sets of genes that function co-ordinately in the biological processes of interest (Gachon et al. 2005). Several constructs have been developed for use in gene transfer to facilitate the generation of herbicide-, insect-, viral-, fungal-, bacterial-, and nematode-resistant plants (Gubba et al. 2002; Hsieh et al. 2002; Jeanneau et al. 2002; Dasgupta et al. 2003; Grover and Gowthaman 2003; Ranjekar et al. 2003; Prins et al. 2008). Transgenes that may affect quality traits of important crops (Paine et al. 2005), and those for antigens and proteins of pharmaceutical importance, have been introduced into transformation vectors.

Agronomically important genes for biotic and abiotic stresses and quality attributes have been the major focus of research on genetic manipulation, with an extensive range of chimeric genes being introduced into plants (Babu et al. 2003). The majority of transgenes introduced express enzymes that confer novel traits on the respective plants. Proteins lacking enzymatic activity have also been expressed. About 50 important genetically manipulated crops are cultivated in more than 25 countries (Wenzel 2006; James 2008).

1.7.1 Resistance to Biotic and Abiotic Stresses

Biotic and abiotic stresses have a considerable impact on crop growth, development, and productivity throughout the world (Zhao and Zhang 2007). Plant genetic engineering holds the promise of circumventing the problems faced in wide hybridization programs, especially when sources of resistance are not available in taxonomically related species. During the past decade, understanding of the complex molecular events that occur in plant-pathogen interactions has progressed considerably and has provided the opportunity for exploiting the theoretical knowledge and practical skills to generate transgenic plants resistant to pathogens (Grover and Gowthaman 2003). The discovery of abiotic stress-related novel genes, determination of their expression patterns and their roles in adaptation to stress have also provided the foundation for efficient transgenic strategies (Zhao and Zhang 2007).

It is not unexpected that since major crop losses are incited by weeds, insects, viruses, and fungi, increased tolerance to these agents will continue to be a focus of genetic manipulation technology. Transformation of crop plants for increased herbicide tolerance dominated the initial stages of the application of genetic manipulation technology to crop plants. Castle et al. (2006) discussed the ways in which technological advances have been incorporated into agricultural practice and traits introduced into crops such as alfalfa, cotton, maize, oilseed rape, papaya, soybean, and squash, together with the first year of commercialization of the products. Importantly, it is possible to stack transgenes in target plants, conferring tolerance simultaneously to more than one agent.

Behrens et al. (2007) indicated that there has been a rapid increase in the weeds that are tolerant or resistant to the herbicides used with genetically manipulated crops, indicating that such economically important weed management traits may have a finite life. In order to prolong the durability of genetically manipulated

herbicide tolerance, these workers developed a nuclear and chloroplast-encoded herbicide balance strategy based on the inexpensive, widely used, and ecologically safe herbicide, dicamba. Similarly, Soberón et al. (2007) discussed the ways in which the evolution of insecticide resistance by insects threatens the application of effective *Bt* toxins from the soil bacterium *Bacillus thuringiensis* that are employed as bacterial sprays, and *Bt* genes that are introduced into genetically manipulated crops. The natural resistance of insects to insecticides will probably necessitate the use of modified *Bt* toxins in the future. Likewise, Gatehouse (2008) stressed the fact that not all pests are targeted adequately by the *Bt* toxins currently in use. *Bt* toxin expression needs to confer adequate protection against target insects, with plastid transformation being superior to nuclear transformation in this respect. Other approaches for maximizing gene expression include the use of novel *Bt* toxins, gene stacking to effect multiple *Bt* toxin expression and protein engineering.

The exploitation of plant defense proteins, such as α-amylase inhibitors and lectins, is also a possibility; novel approaches include the exploitation of new insecticidal proteins such as those from nematodes, the use of bacterial cholesterol oxidase, and the strong insecticidal effect of avidin. Engineering secondary metabolism of plant defense compounds and of the volatiles emitted by plants, and an RNAi approach to generate double-stranded RNAs are also possibilities. Dudareva and Pichersky (2008) discussed the importance of enhancing plant defense by metabolic engineering of volatile compounds, and suggested that priming crops by planting transgenic plants, that constantly emit defense volatiles, among their nontransgenic counterparts, may provide efficient protection. More needs to be known about the properties of specific plant volatiles in terms of their ability to attract or inhibit insect pests.

The status of virus resistance in transgenic plants has advanced considerably since the initial studies involving coat protein-mediated resistance (Prins et al. 2008). The precise mechanism of coat-protein-mediated resistance is not fully understood. It varies with different viruses, but the procedure has been successful in a range of target plants. Other approaches include replicase-mediated resistance and resistance based on movement proteins. RNA-mediated resistance against RNA and DNA viruses is also discussed, as are nonviral sources of resistance using genetic manipulation, particularly an antibody strategy to induce plants to synthesize similar compounds (plantibodies). Transgene-mediated resistances against viroids have been investigated, a promising approach being the expression of recombinant dsRNA-specific RNases by transgenic plants. Several strategies for virus and viroid resistance have been described in the literature, but only a limited number have progressed past the "proof-of-principle" stage, or small-scale field trials (Prins et al. 2008).

In a critique of the deliverables from genetic manipulation technology, Collinge et al. (2008) emphasized the fact that, to date, very few genetically manipulated disease resistant cultivars have been generated, in contrast to plants tolerant to insect pests using a *Bt* approach, and plants that are herbicide tolerant. Indeed, insect- and herbicide-tolerant plants represent more than 90% of all genetically

manipulated crops generated to date. Weed control exploiting genetic manipulation technology has been facilitated by understanding the biology of herbicide tolerance and the specificity of synthetic herbicides. Similarly, success in the genetic manipulation of insect resistance was based, at least initially, on knowledge arising from the extensive use of the soil bacterium *B. thuringiensis* as a natural insecticide. Since the organisms that cause disease are taxonomically and physiologically diverse with complex life cycles, Collinge et al. (2008) advocated a balance between classical plant breeding and genetic manipulation to generate disease-resistant plants. They concluded that transgenic fungal and bacterial resistances will probably not be introduced into commercial crops in the near future, although progress in the introduction of a barley class II chitinase gene into wheat to confer resistance to *Fusarium graminearum* represents an advancement in engineering fugal resistance (Shin et al. 2008). Plants experience considerable environmental stresses, with drought posing one of the most important constraints for agriculture on a global scale in the near future (Umezawa et al. 2006; Bhatnagar-Mathur et al. 2008). Tolerance to drought, cold, and salinity are often linked, which may facilitate genetic manipulation to combat these natural agents. Mutasa-Gottgens et al. (2009) showed that genetic modification of gibberellin signaling and metabolism significantly delays bolting in crops such as sugar beet, that are vulnerable to vernalization-induced premature bolting and flowering, reducing crop yield and quality. This approach confirms the potential in genetically modifying plants to minimize yield losses due to unfavorable environmental conditions.

1.7.2 Improvement of Quality

Nutritional value, being one of the most important traits for improvement of crop quality, involves enhancement of the content of amino acids and proteins, micronutrients, vitamins, minerals, dietary fiber, sugars, carbohydrates, starch, lipids and oils, which are essential for a healthy diet (Singh et al. 2008). Staple crops, such as cereals, are low in lysine, while proteins of legumes, roots, tubers, and most vegetables are deficient in sulfur-containing amino acids (Sun 2008). Engineering complex synthetic pathways may not be a simple task, as changing one biosynthetic route may have a detrimental effect on other aspects of metabolism.

Attempts have been made to enhance the essential amino acid and protein content of crops (Sun and Liu 2004). Transgenic technology will continue to be used to biofortify crops to increase vitamins and minerals. Engineering of provitamin A to generate “Golden Rice” and “Golden Rice 2” represents a major technological advance in this respect (Ye et al. 2000; Paine et al. 2005). As vegetables and fruits contribute significantly to human nutrition, they represent another important target for genetic modification in terms of tolerance to abiotic stress, nutritional quality, storage products, aromas and, in certain cases, seedlessness (Fraser et al. 2002; Dalal et al. 2006). Larkin and Harrigan (2007) discussed the attempts made to improve the nutritional value of maize and cotton seed, while others focused on

vitamins C (Agius et al. 2003) and E (Chen et al. 2006), particularly on oilseeds (Hunter and Cahoon 2007). Volatiles determine the aromas of fruits, vegetables, and herbs, with genetic engineering being able to ameliorate some of the deficiencies of classical breeding (Dudareva and Pickersky 2008). Tomatoes have been engineered for tolerance to chilling damage (Park et al. 2004), this being of relevance during growth of the plants and during transport of harvested fruit. Delay of fruit ripening and increased shelf-life are also targets for genetic manipulation.

Flavonoids and carotenoids play an important role in human nutrition and health, particularly anticancer activity, and understanding flavonoid and carotenoid biosynthetic pathways has enabled anthocyanins and carotenoids to be up- and down-regulated (Tanaka and Ohmiya 2008). Schijlen et al. (2004) also reviewed the modification of flavonoid biosynthesis in crop plants, while Enfissi et al. (2006) concentrated their attention on the genetic engineering of carotenoids in tomato. Plants have been engineered to produce unusual fatty acids, particularly very long-chain polyunsaturated fatty acids normally found in fish oils and marine organisms (Napier 2007). The longer-term result of engineering complex pathways will be influenced not only by the pathways *per se*, but also by the host plant and physical and chemical parameters. Food allergy is a prevalent medical problem in the western world. Allergen reduction is an important topic for genetic engineering, with RNAi technology being applied to reduce allergens in plants such as apple, peanut, rice, soybean, and tomato (Herman et al. 2003; Gilissen et al. 2005; Le et al. 2006; Chu et al. 2008).

1.7.3 Biopharmaceuticals

Vaccines and antibodies play a major role in human healthcare. The majority of drugs used by humans are derived from plants and have resulted in pharmaceutical companies initiating chemical synthesis of medicinally important compounds (Sharma et al. 1999). However, the full potential of synthesizing compounds has been hampered by production costs and maintaining distribution. The progress in plant transformation has attracted attention in exploiting plants as potential bioreactors or biofactories for the synthesis of immunotherapeutic molecules and recombinant proteins. Plants offer several options for transgene targeting and modification (Warzecha 2008). Indeed, as health care becomes an increasing global issue, the longer-term focus of plant genetic manipulation will be towards the biosynthesis of pharmaceuticals (Zhou and Wu 2006) and other specialty compounds (Fischer et al. 2004, 2007; Yonekura-Sakakibara and Saito 2006). Biofortification of crops with micronutrients is another target for genetic manipulation (Poletti and Sautter 2005). Linked to these goals are issues of biosafety, especially the use of marker genes for antibiotic resistance that are common to many transformation procedures. Davey et al. (2008) presented some of the merits and disadvantages of marker gene technology in the transformation of food crops.

Vaccines such as Hepatitis B surface antigen, Norwalk virus capsid protein, cholera toxin B subunit, Rabies virus glycoprotein, and insulin have been expressed in transgenic plants (Mason et al. 1998; Srinivas et al. 2008), as have immunotherapeutic molecules and industrial proteins, including serum albumin, human α-interferon, human erythropoetin, and murine IgG and IgA immunoglobulins. Oral vaccines synthesized in plants may circumvent some of the limitations of traditional vaccines (Robert and Kirk 2006), especially if vaccines can be synthesized in leafy vegetables that are consumed in the raw state. They will also be cost effective, easy to administer and store, and socioculturally readily acceptable (Lal et al. 2007).

1.7.4 Phytoremediation

Activities, such as intensive mining, agriculture, and military operations, release considerable amounts of toxic heavy metals and organic pollutants, posing a serious threat to living organisms (Cherian and Oliveira 2005). Consequently, there is an urgent requirement to decontaminate polluted environments. Phytoremediation, involving the use of plants and microbes to remove pollutants from contaminated soils, sludge, sediments, groundwater, surface water and waste water, is emerging as a cost-effective and environment-friendly technology compared with conventional methods of remediation (Czako et al. 2006).

Plants harbor highly versatile enzymes such as cytochrome P450 monoxygenases, glutathione S-transferases, glycosyltransferases, laccases, peroxidases, and transporters that detoxify pollutants. Although these enzymes may not completely degrade pollutants, they may form complexes, which can be harvested. In recent years, genetic engineering has been used to introduce key genes to increase the remediation ability of several species. Several genes, such as *merApe9, merB, MT1, MT2, CUP1, gshI, ZAT1, ZntA, arsC* (for heavy metal tolerance), mammalian cytochrome P450 2E1 (*CYP2E1*), *cbn4* (for chlorinated solvents), *CYP1A1, CYP2B6, CYP2C9, CYP2C18, CYP2C19* (for herbicide tolerance), and genes encoding rhamnolipid biosynthesis (for oil degradation), have been overexpressed in transgenic plants (Doty et al. 2000; Dhankher et al. 2002; Lee et al. 2003; Thomas et al. 2003; Cherian and Oliveira 2005; Czako et al. 2006), providing a basis for plant-based phytoremediation.

1.7.5 Floriculture

While food crops will continue to be prime targets for genetic manipulation, ornamentals have featured extensively in genetic manipulation strategies because of the significant contribution of the horticultural industry to the economy of many countries (Tanaka et al. 2005). Ornamentals, especially flower species, are well suited to genetic manipulation. As the end product is not food, it does not

necessitate food safety studies, removing major obstacles for commercialization and reducing the cost of production. Chandler and Lu (2005) tabulated the floriculture crops that have been transformed and those with modified characteristics. The latter include disease resistance, herbicide and freezing tolerances (Pennycooke et al. 2003) and, most importantly, modification of pigmentation following manipulation of the genes for pigment biosynthesis (Lu et al. 2003; Tsuda et al. 2004; Suzuki et al. 2007). Attempts have been made to increase the number of flowers produced and extending the life of cut flowers (Shaw et al. 2002). Early and delayed flowering traits have also been introduced (Baker et al. 2002), together with modification of plant architecture (Zheng et al. 2001) and stature (Aswath et al. 2004). The importance of gibberellic acids in controlling plant height in agriculture, horticulture, and silviculture is well recognized (Radi et al. 2006). Dwarf plants may be preferred in amenity planting because of their resistance to unfavorable weather conditions. In this respect, ectopic expression of a gibberellin 2-oxidase from oleander (*NoGA2ox3*) in *Nicotiana tabacum* resulted in dwarf plants (Ubeda-Tomás et al. 2006). Subsequently, Agharkar et al. (2007) demonstrated that genetic manipulation of gibberellin biosynthesis genes can improve the quality of turf grass by increasing the number of vegetative tillers, enhancing turf density under field conditions. Likewise, in order to demonstrate proof of principle and the application of a genetic engineering approach, Dijkstra et al. (2008) overexpressed a gibberellin 2-oxidase gene (*PcGA2ox1*) from *Phaseolus coccineus* to enhance gibberellin inactivation and to induce dwarfism in *Solanum* species. The ability to engineer plant stature through a genetic engineering approach should be of interest to the ornamental industry.

Fragrance will receive more attention (Xiang et al. 2007), since many plants have lost their traditional perfumes through classical breeding. Several approaches have been evaluated to alter scent by genetic modification, as in petunia (Lücker et al. 2001) and carnation (Lavy et al. 2002). However, even though the transgenic plants synthesized more volatiles, the latter could not be detected by humans. In contrast, Zuker et al. (2002) generated carnations with altered floral scent that could be detected by humans, but the resulting plants also had severe alteration in flower color. More recently, Lücker et al. (2004) demonstrated the possibility of modifying the flower fragrance profile by metabolic engineering of tobacco plants using three monoterpene synthases from lemon. These investigators stressed the difficulty of genetically modifying scent because of the need for multigene engineering. Flavonoids and carotenoids are important not only in nutrition and healthcare, as already discussed, but also in flower pigmentation (Nakatsuka et al. 2007b; Tanaka and Ohmiya 2008). Modification of flower color has always been one of the greatest challenges in floricultural plant breeding, since certain colors are difficult to achieve in some species. However, in some cases, genetic manipulation has enabled changes to be made to pigmentation, where classical breeding has failed, by introducing genes from other species and modifying the anthocyanin, carotenoid, or flavonoid biosynthetic pathways. This approach has enabled the generation of purple carnations (Fukui et al. 2003) and blue roses (Potera 2007).

1.8 Risks and Concerns

As with any new technology, there are uncertainties regarding the deployment of genetically engineered plants. There is an increasing concern that insect pests have the capacity to develop resistance against transgenes introduced into plants, or that transgenic properties may be transferred to insects, viruses, and bacteria. Apprehension has also been raised concerning the introgression of transgenes into wild relatives of genetically modified plants and the development of superweeds resulting from introgression of herbicide resistance from transgenic plants to weeds (Sharma et al. 2001, 2002). Transgenic plants may also affect nontarget species and the environment. Food biosafety research has also focused on toxicity and allergenicity of transgenic products.

Although concerns for ecological safety and the human well-being have led to mistrust over the application of genetic manipulation technology, many of these fears appear unsubstantiated or based on misinformation (Stewart et al. 2000). A concerted effort must be made to identify valid concerns and risks, and to provide reliable information to the public. The advent of plant genetic manipulation in vaccine production and quality improvement will increase the emphasis on consumer health benefits, which may facilitate, in turn, acceptance of the use of genetically engineered foods. Active participation of researchers from the fields of biotechnology, ecology, and nutritional sciences may be essential to better determine the biosafety of transgenic plants (Stewart et al. 2000).

1.9 General Conclusions

Modern agricultural biotechnology has been one of the most promising developments in recent years (Sharma et al. 2002). Major advances in understanding gene structure and expression have made significant contributions to the assembly of genes and their regulatory elements for plant genetic engineering. Likewise, progress in DNA delivery technologies has facilitated the introduction of novel genes into a wide range of plants. A common restriction to gene introgression into many crops is the recalcitrance of these plants to express their totipotency in culture. However, the exploitation of procedures that by-pass the requirement for extensive in vitro manipulations should eliminate some of these difficulties. Currently, genetic engineering is not a routine plant breeding tool (Arias et al. 2006), but is an important adjunct to classical breeding (Shewry et al. 2008).

World food supplies will demand more intensive crop production, despite a reduction in available agricultural land because of deterioration of soil quality, drought, climatic change, disease, and political unrest. Farmers will demand more value per unit of agricultural land. Genetic engineering, when used in collaboration with traditional or conventional breeding methods, will be able to increase crop production, increase resistance to major pests and diseases, develop tolerance to adverse weather conditions, improve the nutritional value of some foods, and

enhance the durability of products during harvesting or shipping (Sharma et al. 2002). Reduced use of agrochemicals will have less environmental impact. In the future, agriculturally important traits must satisfy not only the requirements of farmers, but also the availability of materials from researchers, governments, distributors, processors, and the opinions of the public (Castle et al. 2006).

Discussions on transgenic crops have placed undue stress on risk assessment, overshadowing potential advantages (Sharma et al. 2002). The issues relating to genetically modified plants, especially food crops, have been analyzed from a scientist's perspective (Lemaux 2008). These issues are not only complex, but are often aggravated by personal opinions, especially by those members of the public who have limited understanding of plant breeding and gene technology. The rapid escalation of increasingly stringent biosafety regulations regarding transgenic plants or food, in the absence of any scientifically proven genetic risk, is most likely to limit application of transgenic research to meet either the production of sustainable staple foods or the alleviation of poverty (Sharma et al. 2002). Moving crop production from one region to another will influence global trade patterns; legislation and the perceived risks of genetically engineered crops will also affect exploitation of these crops (Singh et al. 2006).

What remains clear is that changes in the genetic complement of those plants that contribute to our food supplies are primarily the result, to date, of sexual hybridization. Genetic engineering provides a precise approach to effect genetic modification over a much reduced time-scale. The safety of genetically engineered plants and those generated by conventional breeding needs to be evaluated on a case-by-case basis (Lemaux 2008). Condemning biotechnology for its potential risks without considering the risks associated with prolonging human misery caused by hunger, malnutrition, and infant mortality is unwise and unethical. The global community must endeavor to remain focused on the target of assuring food for all, and cannot afford to be philosophical and elitist regarding any part of a possible solution, including agricultural biotechnology (Sharma et al. 2002).

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Chapter 2
Explants Used for the Generation of Transgenic Plants

A. Piqueras, N. Alburquerque, and K.M. Folta

2.1 Introduction

The objective of this chapter is to discuss the types of explants more frequently used in the currently published transformation protocols as well as the morphogenic pathways selected for the regeneration of the transgenic plants.

The process of plant genetic transformation can be divided into three phases: (1) foreign DNA transfer into the plant genome, (2) regeneration of the transformed explant into a normal plant, and (3) selection of transgenic plants and confirmation of their transgenic nature. To develop efficient transformation protocols both in herbaceous and woody plants, different explants of distinct morphogenetic potential have been used. For example, hypocotyls, cotyledons, leaves, stems, and roots are all used as starting materials for transformation and regeneration. The following chapter is divided into two principal parts: the first discusses explant selection and utilization in a series of herbaceous crops and the second reviews explant usage in select woody species.

In herbaceous plants, leaf segments are the preferred explants. Organogenesis and somatic embryogenesis, the two more important morphogenetic alternatives in plant tissue culture (Piqueras and Debergh 1999), have been used depending on the recalcitrance of the selected plant. Adventitious shoot regeneration is most frequently used for transgenic shoot regeneration followed by direct somatic embryogenesis from explants or embryogenic cultures.

The following sections present a review of the literature regarding explant selection for transformation and regeneration. These foundations should serve as

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an outstanding platform to test new experimental systems, as well as define new protocols to hasten processes in established models.

2.2 Explants Used for the Transformation of Herbaceous Plants

2.2.1 Cereals

For genetic transformation of cereals, various methods of direct and indirect transfer of foreign DNA are used, and their morphogenic calli or immature embryos (with subsequent stimulation of morphogenic callus formation) are normally used as explants. However, most cereal crops are characterized by a low morphogenic potential and that significantly limits their application for genetic engineering (Danilova 2007).

As a general rule, the morphogenic calli are used as explants for genetic transformation of cereal crops. Alternatively, immature embryos can be used with subsequent initiation of morphogenic callus formation. Regeneration is the next and probably the most important step in genetic transformation of plants. The overall efficiency of transformation greatly depends on the regeneration potential of explants. Much work is being done in many scientific centers throughout the world to increase the regeneration potential of cereal crops. New methods and approaches are tried to widen the range of transformable crops and increase the regeneration potential of calli used. In most cases, the regeneration potential depends on explant type, genotype, and the composition of the cultivation medium (Rout and Lucas 1996; Cheng et al. 2003; Eudes et al. 2003). Traditionally for induction of morphogenesis in vitro, the phytohormones and synthetic hormone-like regulators were used that belong to the auxin family in different combinations with cytokinins (for example BA). Among the most commonly used inducers are 2,4-D-picloram (4-amino- 3,5,6-trichloropicolinic acid) and dicamba (3,6-dichloroanisic acid) (Bahieldin et al. 2000) .

2.2.2 Brassica

Various methods used for Brassica transformation and the factors affecting transformation efficiencies have been reviewed by Poulsen (1996). *Agrobacterium tumefaciens*-mediated transformation is most widely used for Brassica and it is generally quite efficient and practical for most species in the genus. Although seedlings parts, such as hypocotyls, cotyledons, and cotyledonary petioles, are the most common explants, pieces of flowering stalks also regenerate well (Christey and Earle 1991). Flowering stalk explants are less convenient to obtain and more subject to contamination, but have some advantages, particularly when the supply of seeds of a particular genotype is limited (Metz et al. 1994). However, there is still

a need for developing efficient transformation methods to overcome genotype dependency (Cardoza and Stewart 2004).

2.2.3 Cassava

The plant tissue types used for transformation of cassava include shoots induced by organogenesis (Siritunga et al. 2004; Puonti-Kaerlas et al. 1997) and germinating somatic embryos. Direct shoot induction from cotyledons of somatic embryos has been used in both biolistics (Zhang and Puonti-kaerlas 2000) and Agroinfection (Msikita et al. 2002). However, plant regeneration efficiency is highly variable (5–70%) and genotype dependent (Zhang and Puonti-kaerlas 2000). As a result of these cultivar-dependent differences, a variety of tissues including axillary buds (Puonti-Kaerlas et al. 1997), apical leaves (Siritunga et al. 2004), and floral meristems (Woodward and Puonti-Kaerlas 2001) have been used, as various groups found differing success with the various explants from different cultivars.

2.2.4 Potato

Many protocols used now are based on a two-stage regeneration and transformation using stem and leaf explants developed by Visser et al. (1989). The relative ease of shoot regeneration from different tissues of potato (e.g., stem section, leaf, petiole, and tuber disk) conditions the development of the systems used for transformation of this species. At the present moment, most protocols use a two-step regeneration procedure with a callus induction stage followed by a shoot outgrowth stage. The callus stage is minimized to prevent the high incidence of somaclonal variation reported in potato (Heeres et al. 2002). This initial stage is facilitated by treating the explant with zeatin or zeatin riboside in combination with low levels of NAA or IAA. The second stage has the zeatin level reduced by 20% and the auxin level reduced by a factor of 10, plus the addition of gibberellin to stimulate shoot elongation. Usually regeneration rates are high and after 10 weeks of culture ten shoots per explant is a common result.

2.2.5 Sugarcane

Both embryogenic calli (Arencibia et al. 1998) and meristematic tissues from micropropagated plants (Enríquez et al. 1998) have been used as explants for the transformation of sugarcane. The production of sugarcane transgenic plants by agroinfection has been achieved by combining several tissue culture procedures, particularly the use of young regenerable material characterized by the presence of

actively dividing cells competent for agroinfection, pre-induction of the regeneration capacity and treatments to improve the adhesion of *Agrobacterium* during cocultivation (Arencibia et al. 2000).

2.2.6 Banana

Several types of explants have been used for banana transformation (Gómez-Lim and Litz 2004), the most frequently used are embryogenic cultures (Khanna et al. 2004). These cultures are usually induced from immature male (Escalant et al. 1994) and female flowers (Grapin et al. 2000). Wounded meristems of in vitro plantlets have been used for banana transformation as well (May et al 1995). Although transformed plants were regenerated, this procedure has not been widely used because of low transformation rates and chimeras.

2.2.7 Carnation

Stem sections were the first explants used for carnation transformation (Lu et al. 1991); thin sections of node explants have been also used for carnation transformation (Nontaswatsri et al. 2004). So far, the most reproducible and efficient explant used for carnation transformation has been the leaf base of micropropagated plantlets (Firoozabady et al. 1995; Van Altvorst et al. 1995; Kinouchi et al. 2006). By using the leaf base from in vitro grown shoots three cultivars representing three major commercial carnation groups have been transformed in what could be considered a proof to the cultivar independence of this method, transformation efficiency was high and fully transformed carnation plants were produced without chimerism (Firoozabady et al. 1995; Van Altvorst et al. 1995). An example of carnation regeneration is presented in Fig. 2.1.

2.2.8 Tomato

Tomato (*Solanum lycopersicum*) was one of the first crops for which a genetic transformation system was reported involving regeneration by organogenesis from *Agrobacterium*-transformed explants. Commonly, cotyledons from seeds of different tomato lines have been chosen as explants (Frary and Van Eck 2005; Sun et al. 2006; Qiu et al. 2007). With this procedure several tomato cultivars transformed obtained transformation efficiencies that range from 10 to 14% (Van Eck et al. 2006).

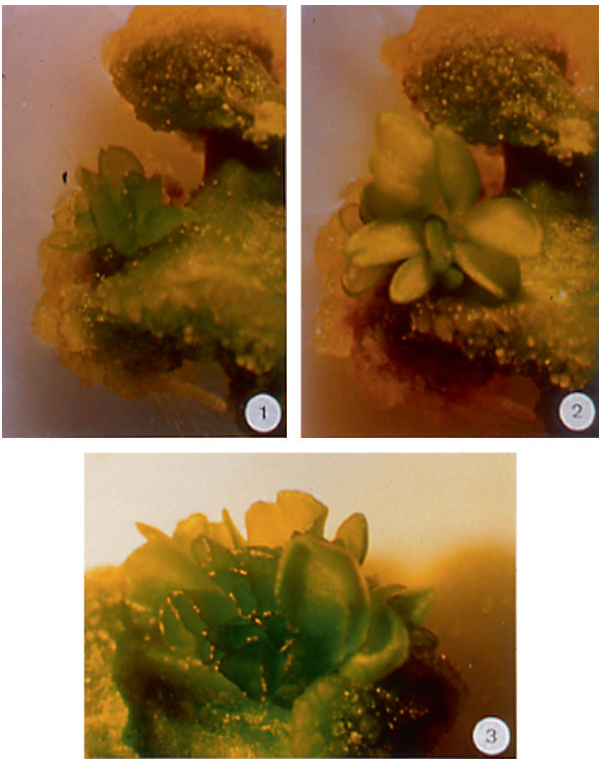

Fig. 2.1 Sequence of morphogenic events leading to adventitious bud regeneration at the leaf base of carnation. (**a**) Emergence of meristemoid, (**b**) Initial shoot cluster formation, (**c**) Developed adventitious shoot

2.2.9 Soybean

Transgenic soybean [*Glycine max* L. Merr] plants have been produced by different methods using explants as embryogenic suspension cultures, proliferating meristems, immature cotyledons, or shoot and axillary meristems from mature cotyledons. Microprojectile bombardment has been used to transform embryogenic suspension cultures and shoot meristems (McCabe et al. 1988; Parrott et al. 1989a; Finner and McMullen 1991; Rech et al. 2008). *Agrobacterium*-mediated transformation methods have been developed with the rest of target tissues (Aragao et al. 2000; Ko et al. 2003, 2004; Liu et al. 2004). The criteria for choosing the type of explants to transform are strongly influenced by the genotype; for instance, the embryogenic response varied with genotype (Parrott et al. 1989b). In a recent work, Cao et al. (2008) have found that there are significant differences among soybean genotypes in their susceptibility to *Agrobacterium rhizogenes* when germinated seedlings have been infected.

2.2.10 Alfalfa

Although different transformed plants of alfalfa (*Medicago sativa* L.) have been obtained by different methods (Samac and Temple 2004), the use of leaf explants of a highly regenerable genotype infected with *A. tumefaciens* followed by induction of somatic embryos have allowed the recovery of transgenic alfalfa plants with extremely high efficiencies (Samac and Austin-Phillips 2006). Samac (1995) reported that transformation at the whole plant level is germplasm dependent, while in tissue culture the bacterial strain used is the critical variable for successful transformation. Other authors (Desgagnes et al. 1995) found a high influence of the genotype, the expression vector and the bacterial stain on the ability to produce stable transgenic material by the method described earlier.

2.2.11 Sunflower

Sunflower (*Helianthus annuus* L.) is considered one of the most difficult species to be genetically transformed because its competent cells for regeneration are not able to be transformed. To overcome this problem, different approaches have been reported such as the origin of the explants, the transformation vectors or systems, and even combinations of approaches. Schrammeijer et al. (1990) developed a routine *A. tumefaciens*-mediated transformation protocol using meristems from late-stage embryonic tissues as efficient explants with low transformation efficiencies. Later, other groups have reported changes in the procedure that have allowed improvement of the efficiency or reduction of chimeral shoot and plant production derived from this kind of explant (Knittel et al. 1994; Rao and Rohini 1999; Molinier et al. 2002).

2.2.12 Cucumber

Cucumber (*Cucumis sativus* L.) transformation has been approached with different kinds of explants using *A. tumefaciens*. Cotyledons (Chee and Slightom 1991; Tabei et al. 1998), hypocotyls (Trulson et al. 1986; Nishibayashi et al. 1996), or petioles (Raharjo et al. 1996) have been used as explants. Embryogenic callus tissues have been bombarded with microprojectiles coated with several plasmid DNAs (Chee and Slightom 1992). One method, which has advantages like fast and efficient plant regeneration from a wide range of genotypes, consisted of producing direct regeneration from leaf microexplants selected on kanamycin-containing medium. The transformation efficiencies varied from 0.8 to 6.5% depending on the genotype and the construct (Yin et al. 2005). Other method involves regeneration from a long-term established embryogenic suspension culture, obtaining higher transformation efficiencies (from 6.4 to 17.9%) (Burza et al. 2006).

2.2.13 Eggplant

Different authors have developed regeneration and transformation procedures to transform eggplant (*Solanum melongena* L.) using seedling explants like hypocotyl, epicotyl, and node segments as well as cotyledon segments (Arpaia et al. 1997; Magioli et al. 1998), leaf disks (Yadav and Rajam 1998), or roots (Franklin and Sita 2003). When an *Agrobacterium*-mediated transformation protocol was used with cotyledons, hypocotyls and leaves from two eggplant genotypes high transformation efficiencies were reported (Van Eck and Snyder 2006).

2.2.14 Melon

In melon species, the transformation frequency is very low due to the production of "escapes" (Guis et al 1998; Galperin et al. 2003). In previous studies, transgenic plants were generated using adventitious shoot organogenesis. To reduce the problem of "escapes," an alternative regeneration system that can enable transformation was developed, and several groups reported the production of somatic embryos from melon cell suspension cultures (Guis et al. 2000). Published protocols for melon genetic engineering use the process of organogenesis (Dong et al. 1991). Although this sometimes leads to problems, such as abnormal embryos, the liquid culture system is considered very useful for the efficient selection of transformed tissues, as whole explants absorb antibiotics more easily when suspended in liquid media than when cultured on solidified media. Embryogenesis is also a useful regeneration system for transgenic research because none of the transgenic plants are chimeric (Asaka-Kennedy et al. 2004). The efficiency of embryogenesis in melons is closely related to the genotype (Oridate et al. 1992).

2.2.15 Strawberry

Strawberry transformation has been reported for many genotypes, although confined to diploid wild species (e.g., *Fragaria vesca*) and cultivated octoploid varieties (*Fragaria* x *ananassa*). Most of the transformation reports generate transgenic plants from *Agrobacterium*-mediated transformation of leaf disks or cut leaves (El Mansouri et al. 1996; Passey et al. 2003; Landi and Mezzetti 2005). In some cases petiole segments are particularly prolific (Folta et al. 2006). New systems in strawberry transformation are being constantly retooled or developed because of strawberry's position as a functional system to test gene activity in the valued Rosaceae family (Shulaev et al. 2008). A demonstration of regeneration from diverse tissues is shown in Fig. 2.2.

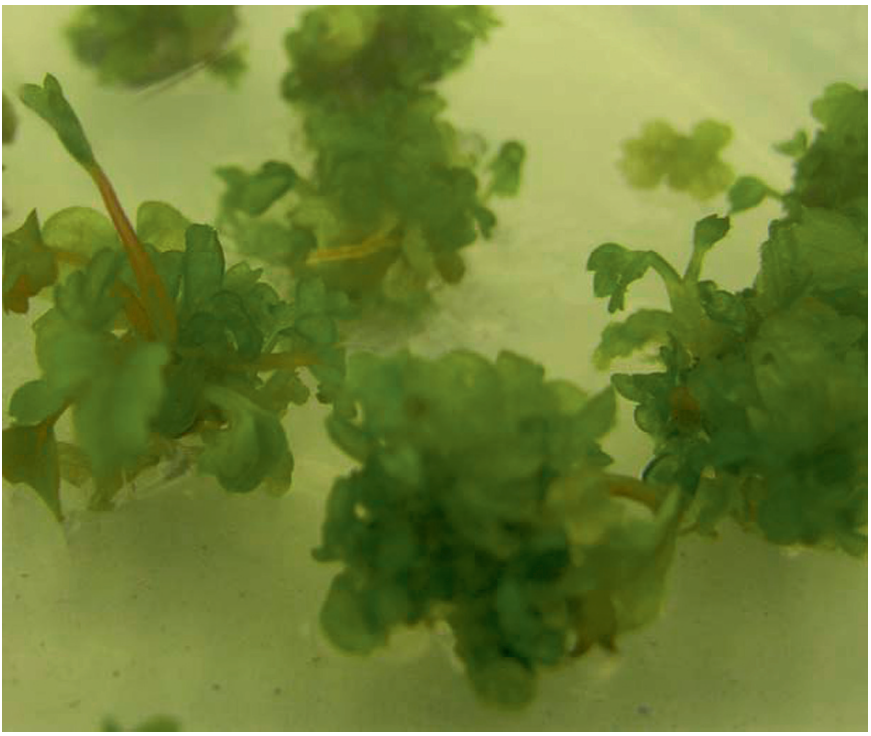

Fig. 2.2 Shoot emergence from diploid strawberry leaf explants. Culture conditions have been optimized for prolific regeneration from a number of diploid accessions, providing an outstanding functional genomics resource for rapid elucidation of gene function

2.3 Explants Used for the Transformation of Woody Plants

Woody plant transformation has become a central area of interest, for valuable forest products, both economical and ecological. Studies of lumber-crop transformation are driven by a need for improved lumber, food, paper, fuels, and other materials that are derived from tree crops. In addition, valuable nutritious fruit and nut products are borne from woody perennials. Lumber, biomass, food, and ecologically intended tree crops will benefit from in vitro propagation or development of genetically enhanced germplasm. Interest has been piqued since the unveiling of the *Populus* genome. There is now extensive interest in transformation of tree crops to validate in planta the findings of genome sequencing and functional genomics studies. The generally long juvenile periods and dormancy issues make breeding efforts and crop improvement strategies difficult and arduous. The need for improved woody species is being met in two ways. First, direct engineering and deployment of tree crops featuring genes of interest permit favorable traits to be directly introduced into production scenarios. A few such examples include the

notable engineering feats of introducing papaya ringspot virus resistance into papaya (reviewed in Tripathi et al. 2008) and plum pox virus resistance into stone fruits (Ravelonandro et al. 1997). Transformation has also been used to engineer rootstocks to reduce the time before scion reproductive competence. The goal is to introduce genes associated with inducing the floral transition so that traditional breeding techniques could be implemented. Such milestones have been met in poplar (Hsu et al. 2006) and citrus (Nishikawa et al. 2007). The challenge to these processes is the engineering itself, as many tree species are recalcitrant to genetic manipulation.

For generation of transgenic materials, it is necessary to regenerate organs and/or embryos using fairly standardized protocols. In these cases, explant selection is central to successful transformation and regeneration. The tissue chosen, as well as the health status and developmental state of the donor plant are critical to the success of adventitious shoot production. In just about all cases callusing and shoot production was greatly accelerated by specific growth regulators, viz. thiadiazuron, discussed elsewhere in this volume. This part of the chapter focuses on economically important woody crops, primarily those used in fruit and forest industries.

2.3.1 Almond

The almond (*Prunus dulcus* Mill.) literature presents several complementary studies of transformation or regeneration, but relatively efficient protocols that combine the two have only been recently developed. *Agrobacterium*-mediated transformation (albeit without regeneration) of almond leaf disks was reported in 1995 (Archilletti et al. 1995). Successful transformation and regeneration of almond was reported 4 years later. Miguel and Oliveira (1999) used the four most recent fully expanded leaves from 3-week-old shoots in culture as explants. A subset of the leaves received a pretreatment on the callus induction media for 3–4 days before wounding. These tissues were cut with a scalpel dipped in bacterial suspension. The efficiency of this approach was low, but successful, and could be greatly increased with addition of acetyosyringone to cocultivation medium (Costa et al. 2006). A contemporaneous study carefully examined regeneration conditions for two major cultivars (Ainsley et al. 2000) leading to higher regeneration efficiency. This study also used young leaves from in vitro grown plantlets, cutting them into 5-mm^2 pieces. Additional reports examined the regeneration from embryonic cotyledons under different culture conditions and growth regulators, greatly improving efficiency (Ainsley et al. 2001b). A complementary study from the same group examined transformation protocols (Ainsley et al. 2001a) but did not report regeneration. Efficient transformation and regeneration has been reported using the same explants – the fully expanded leaves from 21- to 28-day-old micropropagated shoots, cut across the midrib (Costa et al. 2006; Ramesh et al. 2006).

2.3.2 Apple

Generation of adventitious shoots from apple (*Malus* x *domestica* Borkh.) leaf explants has been documented since 1983 (Liu et al., 1983a, 1983b). Genetic transformation of apple tissue was reported two decades ago using leaf explants (James et al. 1989; Maheswaran et al. 1992), and stable integration and segregation of transgenes were later reported (James et al. 1994, 1996). In these early studies *Agrobacterium*-mediated transformation was used with leaf explants, and the effects of explant age, orientation, and genotype were explored (Debondt et al. 1994; Yepes and Aldwinckle 1994; Puite and Schaart 1996). Regeneration from embryonic cotyledons and axes was reported (Keulemans and Dewitte 1994). The effect of various growth regulators was also assessed on leaf explants (Yancheva et al. 2003) as were the effects of acetyosyringone and explant pretreatment (Seong and Song 2008). Internodal seedling explants were shown to be especially amenable to transformation, particularly when etiolated (Liu et al. 1998). Another study utilized stem microcuttings and *A. rhizogenes* to create chimeric apple trees that later yielded transgenic plants (Lambert and Tepfer 1992). Leaf explants have also been successfully used for particle bombardment (Gercheva et al. 1994).

2.3.3 Apricot

Apricots (*Prunus armeniaca* L.) are less routinely transformed than other *Prunus* counterparts such as plum (reviewed later in this chapter), but have benefited from careful studies that have defined optimal conditions. Early studies defined the conditions of regeneration, and sometimes transformation, from a variety of explant types. Transfer of genes using *Agrobacterium* has been accomplished using embryonic cotyledons, leaf disks, and somatic embryos as initial culture material (Machado et al. 1994). Efficient regeneration was achieved from young in-vitro-derived leaves (typically the first four from 21-day-old in vitro plants), but was highly dependent on genotypes and cytokinin and gelling agent used (Perez-Tornero et al. 2000; Burgos and Alburquerque 2003), and could be accelerated with ethylene inhibitors (Burgos and Alburquerque 2003). A series of studies have increased the efficiency of transformation and regeneration of the "Helena" cultivar using a series of selection strategies, growth regulators, and culture conditions, but the explant source has remained unchanged (Petri et al., 2004, 2005a, 2005b, 2008a). Emerging shoots from an apricot explant are presented in Fig. 2.3.

2.3.4 Blueberry

Successful transformation of leaf materials from a blueberry hybrid (*Vaccinium corymbosum* x *V. angustifolium*) was first achieved by Graham et al. (1996). Since

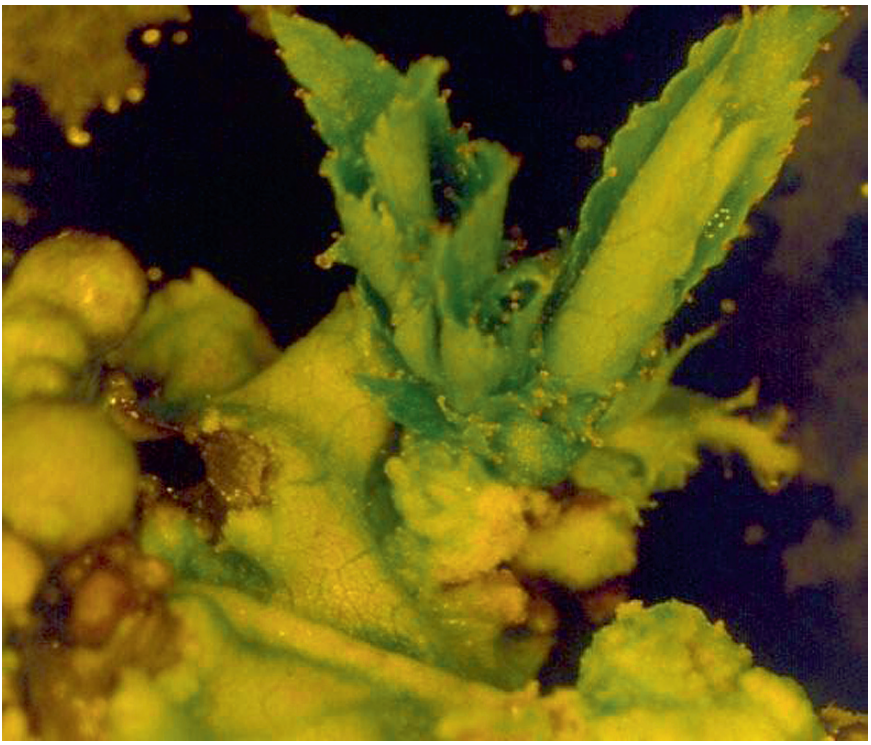

Fig. 2.3 Adventitious bud regeneration on a leaf segment base of apricot cv. Helena

then, multiple studies have examined gene transfer to cut leaf sections from multiple *V. corymbosum* genotypes raised in vitro (Cao et al. 1998; 2003; Sink and Song 2004; Song and Sink 2004). One of the factors affecting transformation efficiency is explant age, as those removed from recently transferred source material typically performed better (Cao et al. 1998). Song and Sink (2004) report successful transformation and regeneration using leaf explants when the petiole is removed and the distal third of the leaf blade is discarded. This latter protocol has been successfully implemented in testing various promoters in this valuable, yet recalcitrant crop (Song et al. 2008).

2.3.5 Birch

The first reports of birch transformation were obtained in Japanese white birch (*Betula platyhphylla*). Here leaf disk explants were transformed and regenerated with reasonable efficiency (Mohri et al. 1997). A study in silver birch (*Betula pendula*) tested regeneration in leaf, internodal stem segments, and nodal stem

segments, and the results showed that all were capable of producing adventitious shoots at a high (> 90%) rate (Lemmetyinen et al. 1998). Transformation was performed on explants that were precultured and wounded before cocultivation (Lemmetyinen et al. 1998). Cultivated callus and shoots were used for biolistic-assisted transformation of silver birch, producing stable plantlets (Valjakka et al. 2000).

2.3.6 Citrus

There is a desire to transform both the scions and the rootstocks of major citrus cultivars, as a changing spectrum of pests, pathogens, and production challenges forces new and rapid innovation. Many genotypes have been regenerated, namely sour orange (*Citrus aurantium* L.), sweet orange (*Citrus sinensis* L. Osbeck), grapefruit (*Citrus paradisi*), mandarin (*Citrus reshni* Hort. ex Tan.), alemow (*Citrus macrophylla* Wester), and the hybrid Troyer citrange (*Citrus sinensis* [L.] Osbeck) among others. The development of transgenic plants was performed by generating transgenic shoots that could be grafted to seedling rootstocks. These shoots arose from internodal stem segments from 5-week-old seedlings, or on the cut end of epicotyls treated with *Agrobacterium* (Pena et al. 1995). In one particular study epicotyl segments from germinated seedlings were cultured in darkness, horizontally, and robust shooting occurred on the basipetal end, arising from the cambial region (Bordon et al. 2000).

2.3.7 Cherry

Cherry is also considered to be recalcitrant to transformation and regeneration. A number of reports have demonstrated the ability to regenerate shoots of several sweet cherries (*P. avium*) grown in vitro (Grant and Hammatt 2000; Bhagwat and Lane 2004; Feeney et al. 2007; Canli and Tian 2008). Bhagwat and Lane (2004) tested a series of explants in two cultivars, comparing the furled leaves at the apex to the expanding leaves to mature leaves, with and without perpendicular wounds across the midrib. The results showed that regeneration occurred only in the upper expanded leaves that were wounded. An evaluation of several explant types was performed by Feeney et al. (2007). In this study explants arose from orchard trees, demonstrating that organogenic callus could be derived from ex vitro materials. Wounding was also advantageous in regeneration of orchard and in vitro tissues. The study by Feeney et al. (2007) also indicates that callus formation becomes less robust as explants are located near the bottom of the plant, as those proximal to the shoot tip performed better. In sour cherry (*P. cerasus* L.) the cultivar "Montmorency" was used for regeneration and transient expression, again using leaf explants with cuts perpendicular to the midrib, much like in apricot (see earlier). These

protocols were the basis of transformation of the "Montmorency" cultivar, where additional culture conditions were evaluated (Song and Sink 2006).

2.3.8 Eucalyptus

The first report of transgenic eucalyptus (*Eucalyptus globulus* Labill.) trees describes *Agrobacterium*-mediated transformation of wounded seedlings (Moralejo et al. 1998). The process was also described with careful detail using *Eucalyptus camaldulensis* hypocotyl segments and organogenesis from callus (Ho et al. 1998). Transgenic plants bearing genes for resistance to herbicide and insect larvae were generated from cut hypocotyls and cotyledons of 2-week-old seedlings (Harcourt et al. 2000). Stable transformation was also achieved by sonicating seeds or seedlings in the presence of *Agrobacterium* (Gonzalez et al. 2002), where the most efficient transformation occurred in the intersection of the root and shoot or cotyledons. Particle bombardment of eucalyptus hybrid callus derived from seedling hypocotyls and cotyledons also resulted in successful regeneration of stable transgenic trees (Sartoretto et al. 2002). While the typical goal of a transformation system is to generate stable plants bearing a transgene, other attempts have examined stably engineered tissue to study wood formation (Spokevicius et al. 2005) or cell fate in transformed cambium (Van Beveren et al. 2006).

2.3.9 Kiwi

Successful transformation and/or regeneration was/were reported for several species of *Actinidia*. The first reports appear from *Actinidia chinensis* (Suezawa et al. 1988). Callus was produced from field-grown leaves and then regenerated through cell suspension cultures. Later, Rugini et al. (1991) transformed elite kiwi germplasm, inserting the *rol A, B*, and *C* genes from *Agrobacterium rhizongenes*, with the intent of affecting root morphology and rooting ability. Here leaf disks were used to directly generate roots, approaching 100% efficiency. At the same time other groups found great success from cocultivation of kiwi hypocotyls or stem segments (Uematsu et al. 1991). In a separate study, kiwi hypocotyls were inoculated with *A. rhizogenes*, leading to prolific hairy root production in culture, eventually generating transformed whole plants (Yazawa et al. 1995). The same group later switched to petioles as leaf explants from several cultivars and obtained up to 31% generation of transformed adventitious buds, again using *A. rhizogenes* protocols (Yamakawa and Chen 1996). Leaf disks and petioles were used to install the stilbene synthase gene (Kobayashi et al. 2000). A smaller, less vigorous, faster flowering species (*Actinidia eriantha*) was transformed and regenerated from leaf strips as a potential system for functional genomics (Wang et al. 2006). Transgenic plants have been regenerated from leaf disks of "Hayward" to test effects of a grape MYB protein on plant pigmentation (Koshita et al. 2008).

2.3.10 Larch

Hypocotyls of developing European larch (*Larix deciduas* Mill.) seedlings were used to introduce transgenes via *A. rhizogenes*-mediated transformation. Wounded hypocotyls from 7-day-old seedlings would produce adventitious shoots 4 weeks after transformation (Shin et al. 1994). An alternative approach introduced genes through "embryogenic masses," cultures formed from long-term maintenance of embryogenic tissue derived from embryos isolated from pollinated cones. Transient expression was observed through microprojectile bombardment (Duchesne 1993) into these masses. Stable transformation of somatic embryos was achieved in *L. laricina* (tamarack). These embryos were both from the precotyledonary stage and those with elongating or developed cotyledons (Klimaszewska et al. 1997). Embryogenic masses were also the preferred starting point for transformation of hybrid larch (Levee et al. 1997). Eventually optimization of particle bombardment protocols would yield stable transformants as zygotic embryos from *L. gmelinii* L. were transformed with this method (Lin et al. 2005). The tissues were cultured to callus that was then induced to form shoots with reasonable frequency.

2.3.11 Peach

Among major tree crops, transformation of peach (*Prunus persica* L. Batsch.) has remained difficult. Despite the efficient systems devised for apricot and plum mentioned elsewhere in this chapter, reports of peach transformation are sparse. Peach has been successfully regenerated from in vitro leaves from plant apices (Gentile et al. 2002), mature and immature cotyledons (Mante et al. 1989; Pooler and Scorza 1995), and zygotic embryos (Hammerschlag et al. 1985). A variety of peach explants, including leaf segments, immature embryos, and embryogenic calli, have been transformed via *Agrobacterium* (Scorza et al. 1990) and biolistics (Ye et al. 1994), but routine regeneration of transformed tissue has remained elusive. Only two reports of successfully reproducing stable transgenic plants were reported and indicate a relatively inefficient transformation and regeneration rate (Smigocki and Hammerschlag 1991; Perez-Clemente et al. 2004). The most successful report demonstrated that regeneration could occur from embryo sections, but was poor or nonexistent from hypoctoyls and cotyledons (Perez-Clemente et al. 2004). Padilla et al. (2006) performed a strategic study using *Agrobacterium*-mediated transformation and GFP markers to assess transformation efficiency of various bacterial strains and explants. The study showed that internodes, cotyledons, and embryonic axes were superior to embryonic hypocotyl slices, the choice material for plum. Still, further optimization will be required to make peach transformation and regeneration routine.

2.3.12 Pear

Pear (*Pyrus communis* L.) has been successfully transformed and regenerated using leaf explants and *Agrobacterium*-mediated gene transfer (Mourgues et al. 1996). Various culture conditions and genotypes were tested, using in vitro-derived leaves from recently transferred plantlets, cut perpendicularly across the midrib (Chevreau et al. 1997; Bell et al. 1999; Yancheva et al. 2006). The same protocols were employed by other studies (Reynoird et al. 1999) demonstrating their utility. Regeneration remained an issue in some genotypes and Matsuda et al. (2005) examined other explant sources to improve efficiency. This report used the same cut leaves from in vitro plants, but then also included 0.5-mm sections of axillary shoot meristems. The meristematic tissues proved superior in otherwise recalcitrant cultivars, and the authors noted that the poor regeneration on selection agents in leaf explants arose from a lack of transformation, not an inherent inability to regenerate. Embryonic cotyledons in mature seeds of the Asian pear (*Pyrus betulaefolia*) have also been amenable to transformation and regeneration (Kaneyoshi et al. 2001).

2.3.13 Pine

Stable transformation of conifers dates back over two decades to efforts of Ron Sederoff and colleagues in loblolly (*Pinus taeda* L.) and sugar pine (Sederoff et al. 1986; Loopstra et al. 1990). Despite these early gains, most reports of pine transformation acknowledged only transient expression, and not the generation of transgenic plants. Efficient generation of transgenic plants is limited by the cell division capacity of the explants in these recalcitrant species. Studies in both pine and spruce (noted later in this chapter) demonstrated increased transformation efficiency when embryogenic tissues are used as explants.

White pine (*Pinus strobus* L.) transgenics were efficiently produced using *Agrobacterium*-mediated transformation against embryogenic tissues (Levee et al. 1999). *Pinus radiata* has also been successfully transformed (Walter et al. 1998).

2.3.14 Plum

Transformation of plum (*Prunus domestica* L) is routine and efficient, at least compared to other closely related stonefruits like apricots, almonds, and peaches. Plum transformation dates back almost two decades to reports of *Agrobacterium*-mediated transformation of hypocotyl segments isolated from the embryonic axes in ungerminated seeds (Mante et al. 1991). Here the surface-sterilized seed was split and the hypocotyl was removed and cut into three sections. The radicle and the epicotyl were discarded, and the central portion was used for transformation by

slicing it transversally into several thin (< 1 mm) explants. These protocols have remained generally unchanged, except for a 10× increase that comes from preconditioning explants with growth regulators (Petri et al. 2008b). Similar protocols also work with the Japanese plum (*Prunus salicina*) (Urtubia et al. 2008)

2.3.15 Populus

Populus species and related hybrids have become extremely useful model plants in the study of gene function in woody plants, due in part to the full accounting of genes in a sequenced genome and rich expressed sequence tag (EST) resources. Transformation in *Populus* was first achieved over two decades ago (Parsons et al. 1986), and subsequent studies improved on the techniques. Explants used include internode pieces from 6- to 8-week-old in vitro plants (Deblock 1990), and leaf disks from ex vitro plants (Tsai et al. 1994). Transformation of *P. tremula* (Tzfira et al. 1996) and cottonwood varieties (Han et al. 1997) was accelerated using *A. rhizogenes* against stem segments that would develop adventitious roots with great efficiency. Additional protocols were specifically designed for the *P. trichocarpa* genotype Nisqually-1, the line used for genome sequencing. Here internodal stem explants proved superior to midrib or leaf explants in regeneration efficiency (Song et al. 2006). This study showed that explant selection was critical to the transformation process, both in the discrete tissue used and the age of the explant source plant. Specifically, the fifth to eighth stem internode sections from vigorous 5–6 month-old plants performed best in culture. Recent modifications hasten the process in quaking aspen (*P. tremuloides*) inoculating hypocotyl sections leading to the regeneration of transgenic trees in 3–4 months instead of 6–12 months (Cseke et al. 2007).

2.3.16 Spruces

Low transformation rates in spruces were caused by explant materials with limited competence for cell division. A comprehensive assessment of transformation competence during embryo development optimized parameters of white spruce (*Picea glauca*) transformation by particle bombardment (Ellis et al. 1993). Embryogenic callus, embryos themselves, and seedlings were receptive to the treatment, leading to transformed plants. Experiments using cell suspension cultures of Norway spruce further accelerated efficiency. Norway spruce (*Picea abies*) offers the advantage of a well-studied system with prolific cell growth, excellent culture viability, and strong regeneration potential for embryogenic cultures (von Arnold et al. 1996). Studies in *P. abies* demonstrated that biolistics technologies could be used to introduce transgenes, such as GUS (Duchesne and Charest 1991). Biolistics or *Agrobacterium* have been used to generate transgenic plants arising from somatic embryos (Walter et al. 1999).

2.3.17 Walnut

The transformation of walnut (*Juglans* spp.) via *Agrobacterium*-associated means was originally tested by Polito et al. (1989) when they were able to produce somatic embryos in culture. At the same time, studies at the University of California-Davis were demonstrating that the somatic embryos of walnut could be transformed and regenerated into plants (McGranahan et al. 1988, 1990; Dandekar et al. 1989). As with many hardwoods, the most efficient transformations have been performed on somatic embryos (Escobar et al. 2000; Tang et al. 2000). Somatic embryos themselves have even been reported to produce secondary embryos (Raemakers et al. 1995), appropriate for transformation. Transformation of agriculturally useful transgenes has been reported for Persian walnut (*Juglans regia*) when somatic embryos derived from a repetitively embryogenic lines were cocultured with *Agrobacterium* bearing the gene encoding Bt toxin (Dandekar et al. 1998). An additional study defined the boundaries of transient and stable transformation of somatic embryos in Persian walnut using GFP (Escobar et al. 2000). This visible marker allowed detection of transformed materials that could be subcultured into plants on appropriate media.

2.4 Concluding Remarks

Explant selection is a critical parameter to consider when performing transformation and regeneration experiments. Different explant types often have varying potential for transformation and certainly for organogenesis or development of somatic embryos. Just as a complete test for transformation and regeneration includes a complete assessment of growth regulators, media constituents, and culture conditions, the choice of explant should be a central consideration in the development of transgenic resources.

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Chapter 3
Gene Transfer Methods

Seedhabadee Ganeshan and Ravindra N. Chibbar

3.1 Introduction

The ability to alter the genetic composition of a plant is fundamental to crop improvement and development of new cultivars with desirable characters. Plant breeders have utilized the naturally occurring genetic variability in existing germplasm to develop new lines by sexual hybridization. In the absence of natural variation for a trait, chemical and radiation mutagenesis was used to create genetic variability for use in the development of varieties with desirable traits. In another approach, genes for superior traits in close relatives were identified and recombined by wide hybridization, thereby generating interspecific or intergeneric hybrids between the donor and target species. However, all these chromosome-mediated gene transfers need sexual hybridizations. Sexual compatibility and chromosome pairing are key components for the introgression of a desired trait. To overcome limited sexual compatibility, embryo rescue using in vitro culture techniques was used to induce genetic variability for desirable traits (Raghavan 1986). The development of protoplast culture and somatic cell hybridization was one of the first examples to create genetic variability by asexual means. Furthermore, in vitro culture of plant cells in suboptimal conditions was found to induce genetic variations termed somaclonal variation, subsequently exhibiting an altered phenotype (Larkin and Scowcroft 1981). The *Agrobacterium tumefaciens*-mediated integration of foreign DNA into a cell's nuclear genome and production of a transgenic plant in which the inserted gene was inherited following Mendelian genetics was the ultimate method to create genetic variation across species, irrespective of genetic proximity or sexual compatibility (Otten et al. 1981).

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3.2 Gene Delivery Methods

The availability and the versatility of different plant DNA delivery methods have become even more pertinent in recent years with the availability of gene sequence data and the need for functional analysis of cloned and sequenced genes. Although the existence of a vast depository of sequences of known functions in the databases can be used for the prediction of gene function of unknown sequences based on homology, limitations are often encountered with respect to precisely determining functions of the genes of interest (Sessions et al. 2002). Therefore, the availability of high-throughput gene transfer systems for economically important crop plants has become highly desirable to expedite gene function analysis. While such transformation systems are routine in model systems such as *Arabidopsis*, for many economically important crop plants, extensive effort is still required to achieve routine, high efficiency transformation. It is, therefore, imperative to understand the practicality, usefulness, and versatility of the different gene transfer methods that can contribute to specific transformation projects. This is particularly critical for species which are presently relatively recalcitrant to genetic transformation.

Since the first report of *Agrobacterium*-mediated delivery of genes to produce transgenic plants in the early 1980s, a number of other gene delivery methods have been reported in the literature (Table 3.1). Some of these have been successfully used to produce transgenic plants for commercial applications and/or basic studies to understand plant growth and development. However, there still remains a challenging task ahead to find the best suited transformation method for various plant species. There is also a need to find cost-effective methods for transformation so that laboratories with limited funding resources are capable of conducting such research. Furthermore, due to patenting issues currently covering some of the transformation methods such as *Agrobacterium*, methods need to be developed for availability in the public domain. Notwithstanding these issues, gene transfer to plants overall appears to be simple, but requires careful interphasing of several different systems.

Thus, simply iterated, gene transfer to plants involves the integration of three components, which include a tissue culture system (discussed in Chap. 2), a DNA delivery system, and a vehicle for carrying the DNA to be transferred (Fig. 3.1). An ideal gene delivery method transfers the carrier DNA to a cell with minimum damage to the recipient tissue, allows for stable transgene integration into the recipient genome and sustained cell proliferation of recipient tissue for subsequent regeneration of a transgenic plant. The commonly used gene transfer methods can be classified into several different groups. In this chapter, two broad groups of gene delivery methods will be discussed: (a) biological, and (b) physical. The biological group includes a living organism, such as a bacterium or virus, to deliver a gene to a host cell. The physical methods include direct DNA delivery techniques, which use a chemical alteration or physical force such as pressure or electric discharge to deliver the vector DNA into a host cell. Recently, a third group of techniques, which use a combination of biological and physical techniques to deliver the vector DNA, has been developed. These techniques use the desirable features of both the groups to achieve optimal delivery of vector DNA into host cells. Essentially, the methods

Table 3.1 Summary of important gene transfer methods

Approach	Brief description	Reference
Physical delivery methods		
Bioactive beads	Calcium alginate microbead-immobilized DNA molecules for uptake by protoplasts	Sone et al. (2002), Liu et al. (2004), Murakawa et al. (2008a,b)
Electric discharge	Gold particle acceleration via electric discharge to target cells (ACCELL^{TM} technology)	McCabe and Christou (1993)
Electrofusion	Fusion of protoplasts by electric pulses	Morikawa et al. (1986b)
Electrophoresis	DNA transferred electrophoretically to embryos	Ahokas (1989), Griesbach (1994)
Electroporation	Electric pulses delivered to protoplasts, mesophyll cells, intact tissues	Fromm et al. (1985), Lorz et al. (1985), Morikawa et al. (1986a), Li et al. (1991), Arencibia et al. (1995)
Laser micropuncture	Laser-mediated perforations in cells and tissues allow uptake of exogenous DNA	Guo et al. (1995), Badr et al. (2005), Kajiyama et al. (2008)
Liposomes	Liposome containing DNA taken up by protoplasts	Deshayes et al. (1985)
Macroinjection	Injection of DNA into floral tillers	de la Pena et al. (1987)
Microinjection	Injection of DNA into protoplasts, intact cells such as callus and embryoids	Griesbach (1983), Morikawa and Yamada (1985), Crossway et al. (1986), Griesbach (1987)
Nanoparticles	Honeycomb mesoporous silica nanoparticles containing DNA taken up by protoplasts	Torney et al. (2007)
Particle bombardment	Acceleration of microprojectiles such as tungsten and gold	Klein et al. (1988a), Sanford (1990), Vasil et al. (1991)
PEG-mediated DNA uptake	Protoplasts	Uchimiya et al. (1986)
Pollen	Pollen/plant DNA mixture used for self-fertilization in maize	Ohta (1986)
Pollen tube pathway	DNA applied to cut styles just after pollination and flows along pollen tube to the ovule	Luo and Wu (1989)
Silicon carbide whiskers	Vigorous shaking or vortexing of silicon carbide fibers with DNA and suspension cells, embryogenic callus	Kaeppler et al. (1992), Frame et al. (1994), Petolino et al. (2000)
Somatic cell hybrids	Fusion between protoplasts and generation of somatic hybrids	Carlson et al. (1972), Kao et al. (1974), Wallin et al. (1974)
Biological delivery methods		
Agrobacterium rhizogenes	Carrot disks, tobacco, and morning glory stem segments inoculated	Chilton et al. (1982), Tepfer (1984)
Agrobacterium tumefaciens	Tobacco stem segments, leaf disks	Barton et al. (1983), Fraley and Horsch (1983)

(*continued*)

Table 3.1 (continued)

Approach	Brief description	Reference
Agroinfection	Turnip leaves inoculated with *Agrobacterium* containing viral DNA engineered into T-DNA	Grimsley et al. (1986)
In planta	By-passes tissue culture, and *Agrobacterium* suspension applied by vacuum infiltration or dip to floral parts, meristems, embryo axis	Bechtold et al. (1993), Clough and Bent (1998)
Other microorganisms	*Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* strains engineered with disarmed Ti plasmid and used to inoculate tobacco and *Arabidopsis*	Broothaerts et al. (2005)
Combination of physical and biological methods		
Agrolistics	Combination of biolistic DNA delivery and *Agrobacterium*, wherein *vir*D1 and *vir*D2 genes are delivered biolistically and cause in planta excision of T-DNA	Hansen and Chilton (1996)
Sonication assisted	Plant tissue subjected to short periods of ultrasound in the presence of *Agrobacterium*	Trick and Finer (1997)

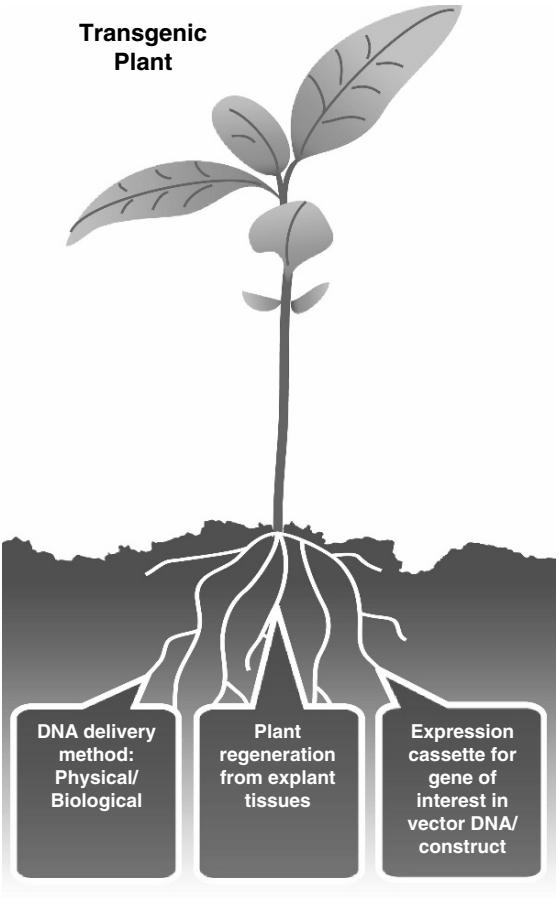

Fig. 3.1 A three-component system required for successful production of transgenic plants. The three components integrate a tissue culture system, a DNA delivery system, and a vector carrying the gene of interest

follow a general plan (Fig. 3.2). The initial step is the DNA delivery to cells or tissues, followed by culture and selection to allow only those cells and tissues having a marker gene (e.g., antibiotic or herbicide resistance gene) to survive and proliferate further. Subsequently, plants are regenerated from the surviving cells, rooted and hardened in the soil. Such primary transformants are thereafter used for molecular analyses for determination of integration and copy number of the transgenes of interest.

3.2.1 Biological Methods

3.2.1.1 Agrobacterium-Mediated Gene Transfer

The first report of a transgenic plant was as a result of *A. tumefaciens*-mediated delivery of foreign DNA (Otten et al. 1981; Barton and Chilton 1983; Fraley and Horsch 1983). Since then, *Agrobacterium* has been used to deliver DNA in several

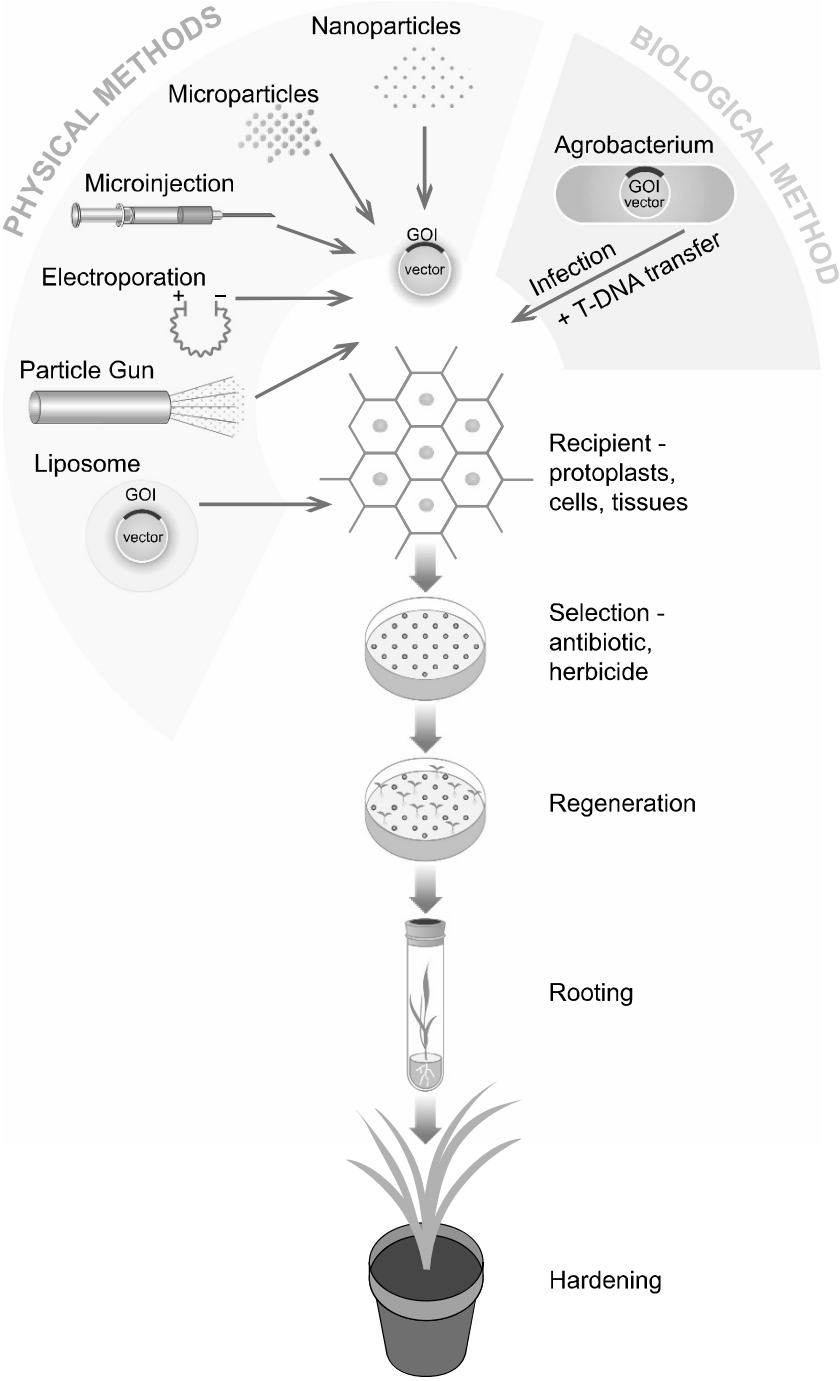

Fig. 3.2 Schematic depiction of physical and biological methods commonly used for gene transfer to plants and the general steps leading to the growth of a putative transgenic plant

plants to understand the basic principles of plant biology and for developing crop cultivars with improved agronomic traits including enhanced crop quality traits. *Agrobacterium* as a genus can transfer DNA to a very broad range of organisms including plants (both monocotyledonous and dicotyledonous angiosperms), gymnosperms, fungi, and recently to human cells (Kunik et al. 2001). Among the five known *Agrobacterium* species, *A. radiobacter* is avirulent, three species are pathogenic and cause crown galls (*A. tumefaciens*, *A. rubi*, and *A. vitis*), while *A. rhizogenes* is responsible for hairy root disease. This chapter will focus on *A. tumefaciens* and *A. rhizogenes*, the two most common species used for DNA delivery to produce transgenic plants.

A. tumefaciens causes crown galls on a large number of dicotyledonous and a limited number of monocotyledonous plant species, and gymnosperms (Levee et al. 1997; Levee et al. 1999). The *A. tumefaciens*-induced tumors can be grown in vitro in simple culture media without the bacterium and any added plant growth hormones (Braun 1941; Braun and Laskaris 1942; Braun and Laskaris 1943). The crown gall disease is caused by the transfer into plant cells of a specific DNA fragment (T-DNA), which originates from a tumor-inducing (Ti) plasmid present within the bacterium. The T-DNA becomes integrated into the plant nuclear genome, and expression of the genes present on the T-DNA gives rise to the crown gall phenotype. The T-DNA carries some of the genes responsible for auxin and cytokinin synthesis, which result in the rapid and autonomous growth of crown gall tissue in the absence of added plant hormones. The other T-DNA genes are responsible for the synthesis of specific amino acids or sugars which are normally not present in plant cells. These plant tumor-specific compounds are collectively known as opines, but classified as octopines, nopaline, agropine, succinamopine, or chrysopine produced by specific Ti plasmids. These opines can be metabolized by the respective *Agrobacterium* strains but not by other soil microorganisms thus creating a niche environment and host strain specificity, which results in a very conducive environment for *Agrobacterium*-mediated genetic modification of plant cells. *A. rhizogenes* causes hairy root plant disease, which is characterized by the rapid proliferation of roots at the infection site. *A. rhizogenes* transfers an Ri plasmid in a manner similar to the Ti plasmid of *A. tumefaciens*.

Agrobacterium-mediated T-DNA transfer to plants is governed by three basic genetic elements. A native Ti or Ri plasmid generally varies in size from 200 to 800 kb (Goodner et al. 2001; Wood et al. 2001) and usually contains one T-DNA region, which is usually about one-tenth (10–30 kb in size) of the total plasmid. In some instances, a Ti plasmid may contain multiple T-DNA regions (Merlo et al. 1980). The first major element is defined by border sequences, which are 24–25 bp imperfect direct repeats flanking and defining the T-DNA region (Zambryski et al. 1982; van Haaren et al. 1988). These border sequences are the only DNA sequences required in *cis* for T-DNA transfer (Zambryski et al. 1983a). DNA present in between the border sequences is transferred to the recipient plant cell's nucleus. The second important element is composed of virulence (*vir*) genes also present on the Ti plasmid but outside the T-DNA. *VirA* and *VirG* located on the virulence region make a two-component regulatory system for controlling

transcriptional activation of the *vir* operons (Stachel and Zambryski 1986). Some of the *vir* genes are critical in the transfer of T-DNA from the bacterium to host cell, while others help in targeting T-DNA to the nucleus and probably to the precise integration site in the host cell for T-DNA. The third important bacterial genetic element comprises the chromosomal genes critical for attachment of the bacterium to the host plant cell (Sheng and Citovsky 1996). Thus, in the vicinity of wound sites in the plant and the release of signal molecules, bacterial cells are chemotactically attracted to the host cells (Hawes and Smith 1989). It has been found that at the wound site release of monocyclic phenolic compounds such as acetosyringone leads to the induction of the *vir* genes (Stachel et al. 1985). It has generally been proposed that at the initial onset of the infection process, the wound signals are perceived by the VirA protein, which activates the *virG* transcription factor by phosphorylation, leading to the upregulation of other *vir* genes (Citovsky et al. 1992). Among these up-regulated *vir* genes, protein product from the *virD2* gene recognizes the imperfect direct repeats of the T-DNA and in concert with the virD1, virD2, virC1 and virC2 proteins cause a nick in the T-DNA strand (Yanofsky et al. 1986; Yanofsky and Nester 1986; Stachel et al. 1987). The virD2 protein covalently binds the 5′ end of the single-stranded T-DNA (Yanofsky et al. 1986; Vos and Zambryski 1989) and the virE2 protein forms a complex with the T-DNA strand for mobilization into the plant cell nucleus (Citovsky et al. 1988, 1989). It is believed that the type IV secretion system, T4SS, of the *vir* system in *Agrobacterium* is assembled by 11 proteins coded for by the *virB* operon and the virD4 protein leading to the channel bridging the bacterial and plant cell wherein the passage of the T-DNA complex occurs (Christie 1997, 2004; Zupan et al. 1998; Zupan et al. 2000). Once the T-DNA complex is within the plant nucleus, it has been suggested that doubling of the T-DNA occurs and there is integration into plant chromosomes (DeNeve et al. 1997) or transient expression of the genes on the T-DNA.

The utilization of *Agrobacterium* as a gene delivery method was further enhanced by the observations that the disarmed T-DNA lacking functional oncogenes can be transferred and integrated into plant genomes to produce transgenic plants (Barton et al. 1983; Zambryski et al. 1983b). The ability of the *vir* genes to act in *trans* resulted in the development of a small, easy-to-handle binary vector system, which contains two replicons, one containing T-region constituting a binary vector and another replicon containing the *vir* genes termed the *vir* helper (Hoekema et al. 1983). The *vir* helper plasmid contained the disarmed T-DNA and was unable to induce tumors. A number of *Agrobacterium* strains containing nononcogenic *vir* (disarmed) helper plasmids have been developed such as LBA4404 (Ooms et al. 1981), GV301 MP 90 (Koncz and Schell 1986), AGL1 (Lazo et al. 1991), and EHA101, 105 (Hood et al. 1986; Hood et al. 1993). The binary vectors have a variety of restriction sites and carry scorable and selectable markers to estimate transformation events and select transgenic tissues, respectively. The host range of *Agrobacterium* defined its utilization as a gene transfer method to produce transgenic plants. Therefore, initial studies focused on host-pathogen interaction to extend *Agrobacterium*'s host range, which was limited to

dicotyledonous plants, as monocotyledons were considered outside its host range. In order to find the factors limiting its infectivity, most of the studies were devoted to identifying the factors for *Agrobacterium* infection and expanding its host range.

3.2.1.2 Agroinfection

Introduction of plant infectious agents via *Agrobacterium* has been defined as agroinfection (Grimsley et al. 1986; Grimsley and Bisaro 1987) or agroinoculation (Elmer et al. 1988). This technique is applicable to molecules that can replicate independent of the plant chromosomal DNA and has been used to deliver viral DNA by two different methods. In the first approach, viral DNA is placed in tandem in the bacterial T-DNA, and systemic spread of the virus occurs in a recipient host plant after inoculation. This technique does not require preparation of nucleic acids or insect vectors. In the second approach, *Agrobacterium* carrying viral nucleic acid sequences can be integrated into the nuclear genome of every cell in a transgenic plant. The first technique, which has been used to introduce genomes of cauliflower mosaic virus (CaMV), was agroinfectious on turnips when placed in the T-DNA, but not when on a different plasmid replicon, suggesting that the infection was not as a result of *Agrobacterium* lysis (Grimsley et al. 1986). Agroinfection as a DNA delivery method has been used to study the basic aspects of virology, recombination, and T-DNA transfer.

3.2.1.3 Virus-induced Gene Silencing (VIGS)

Due to their economic importance as some of the most severe crop-disease-causing entities, plant viruses have been extensively studied to develop resistant crop cultivars. As early as the 1920s and 1930s, it was recognized that certain virus-infected plants became resistant to the same virus or closely related strains of the virus (McKinney 1927, 1929, 1937) and eventually the term crossprotection came to be widely used to describe this acquired resistance as a result of prior exposure to viruses (Fulton 1986). During the same period, the concept of pathogen-derived resistance (PDR) was proposed to genetically engineer resistance against pathogens (Sanford and Johnston 1985; Grumet et al. 1987). Pathogen-derived genes coding for coat proteins, replicases, movement proteins, defective interfering RNAs and DNAs, and nontranslated RNAs have been associated with PDR (Beachy 1997). The concept as such gained credibility with the production of transgenic tobacco plants carrying a gene coding for the tobacco mosaic virus (TMV) coat protein, which delayed symptom development (Abel et al. 1986). The molecular basis for the delay or absence of symptoms was ultimately attributed to post-transcriptional gene silencing (PTGS) (Ratcliff et al. 1997). It is now well established that PTGS is a result of occurrence of double-stranded RNA, first reported in *Caenorhabditis elegans* (Fire et al. 1998) and subsequently in plants (Waterhouse et al. 1998).

In principle, virus-induced gene silencing (VIGS), which was the term first used to describe the recovery of a virus-infected transgenic plant from virus infection (van Kammen 1997), is a result of PTGS (Kumagai et al. 1995). VIGS has been recognized as a powerful tool to down-regulate the activity of specific genes and use in high-throughput functional genomics studies in plants (Baulcombe 1999). Initial experiments involved engineering phytoene desaturase (PDS) cDNA into the chimeric sequences derived from the TMV and the tomato mosaic virus and application of the in-vitro-transcribed viral RNA to *Nicotiana benthamiana* leaves by rubbing (Kumagai et al. 1995), which led to down-regulation of the *PDS* gene. The *PDS* gene has also been used in conjunction with the barley stripe mosaic virus (BSMV) for gene silencing studies in monocotyledonous plants such as barley (Holzberg et al. 2002). Furthermore, more pronounced silencing was possible by inserting 40–60 bp direct inverted repeats into viral vectors for both TMV and BSMV (Lacomme et al. 2003).

3.2.1.4 Other Microorganisms for DNA Delivery

Although *Agrobacterium*-mediated transformation of many plant species is now possible, there still remains one major constraint for commercialization aspects of transgenics relating to the numerous patents involving *Agrobacterium*-mediated transformation methodologies (Roa-Rodriguez and Nottenburg 2003). There has, therefore, been an interest in recent years at the possibility of exploring other microorganisms for the transfer of DNA to plants. Even prior to the advent of such patents or before the understanding of the detailed molecular aspects of the T-DNA transfer to the plant genome, it was shown that crown gall induction property could be transferred from virulent *A. tumefaciens* strains to avirulent strains as well as to *A. rubi*, *A. radiobacter*, and *Rhizobium leguminosarum* (Klein and Klein 1953). It was eventually shown that transfer of the Ti plasmid to avirulent *Agrobacterium* strains or to *Rhizobium* (Hooykaas et al. 1977) and to the bacterium, *Phyllobacterium myrsinacearum* (Veen et al. 1988) caused the tumor formation. In order to explore the feasibility of using such nonagrobacterial microorganisms for transfer of DNA to plants, attempts were made to transfer the disarmed Ti plasmid, pEHA105, to other species of bacteria (Broothaerts et al. 2005). Modified forms of the Ti plasmid were introduced into *Rhizobium* sp. NGR234, *Sinorhizobium meliloti*, and *Mesorhizobium loti* and transformation of plants, such as tobacco, rice, and *Arabidopsis,* indicated GUS activity as well as GUS-positive signals on the Southern blots (Broothaerts et al. 2005). This study has therefore widened the scope of the T-DNA transfer technology to plants. Furthermore, this alternative approach is available under open-source-modeled licenses. Further details regarding this concept of sharing and technology improvement in an open environment are available at Bioforge project (*http://www.bioforge.net*) and Biological Innovation for Open Society (BIOS; *http://www.bios.net*).

3.3 Physical Methods

3.3.1 Liposome-Mediated Delivery

Liposomes are unilamellar phospholipid vesicles ranging in diameter of 0.2–1.6 μm (Olson et al. 1979; Szoka et al. 1980; Jousma et al. 1987) depending on extrusion techniques and measurement approaches. The encapsulation process preserves the structural integrity of small molecules (5–10 kb). The liposomes with the carrier molecules can be fused with the protoplasts using a fusiogenic agent, polyethylene glycol (PEG), or polyvinylalcohol followed by a high calcium ion treatment to promote protoplast fusion (Keller and Melchers 1973). Infection of tobacco mesophyll protoplasts with liposome-mediated delivery of TMV RNA has also been attempted (Nagata et al. 1981). TMV-specific immunofluorescence assay showed the presence of TMV particles in the infected tobacco mesophyll protoplasts. Similar findings were confirmed showing that negatively charged liposomes delivered nucleic acids into protoplasts better than other types of liposomes (Fraley et al. 1982). Liposome-mediated delivery of other viral nucleic acids has also been shown (Caboche 1990). Liposomes were used to transfer a chimeric gene construct encoding chloramphenicol acetyl transferase (CAT) into various plant protoplasts. The marker gene assay showed that this liposome-mediated DNA transfer technique was comparable to PEG-mediated techniques but less efficient than electroporation (Caboche 1990). Liposome-mediated DNA delivery into tobacco mesophyll protoplasts and subsequent regeneration into mature transgenic plants has also been demonstrated. The introduced kanamycin resistance gene was integrated into the host genome and was inherited in a Mendelian fashion (Bellini et al. 1989). The inserted plasmid DNA was integrated into the host tobacco genome in a complex pattern. Liposome-mediated DNA transfer to produce transgenic plants has been tested for only a limited number of plant species, although there are some reports of success, including those encoding cationic liposomes or lipofectin. Cationic liposomes were developed to overcome some of the earlier difficulties associated with DNA delivery into eukaryotic cells and involved synthesis of a cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N, N,N-trimethylammonium chloride (DOTMA), which readily interacted with DNA for delivery to mammalian cell culture lines (Felgner et al. 1987; Felgner and Ringold 1989). Similar cationic liposomes have therefore been used for the transformation of protoplasts from tobacco (Sporlein and Koop 1991) and lentils (Maccarrone et al. 1992), with evidence of transient expression. More recently, DNA encapsulated within a novel cationic vesicle derived from vernonia oil was shown to pass undamaged across isolated plant cuticular membranes (Wiesman et al. 2007). Further studies are required to improve this system and show that indeed physical barriers of the plant cell can be obviated for DNA delivery by this method, possibly not necessitating protoplasts for transformation and regeneration. Generally, the transformation frequencies with liposome-mediated techniques are low compared to other direct DNA delivery techniques. Therefore,

liposome-mediated DNA delivery has not gained widespread attention for extensive studies or production of transgenic plants.

3.3.2 Nanoparticles

With the recent explosion in nanotechnology-based research for addressing biological questions and providing solutions, particularly in medicine and therapeutics, there has been increasing interest in pursuing such approaches in resolving plant biology-related research objectives and questions. Thus, for gene transfer to plants, exploration on the use of nanoparticles would be valuable. It can probably be argued that efforts undertaken with the use of liposome-mediated DNA delivery have already heralded nanotechnology-directed gene transfer technology in plants. Nanoparticles have been defined as materials which span the nanometric size range, with one of the dimensional size range being within a few hundred nanometers (Gonzalez-Melendi et al. 2008), or colloidal polymeric systems, either biodegradable or nonbiodegradable, of less than one micrometer in size (Brigger et al. 2002). In animal cells and tissues and for drug therapy, the use of nanoparticles is being viewed as effective carriers for delivery of molecules (Yih and Al-Fandi 2006), circumventing degradation if taken orally or delivered unprotected by other means, and also allowing more targeted delivery and release for drugs (Brigger et al. 2002). With the extensive research to develop delivery systems in drug therapy, there has been similar interest for targeted and controlled DNA delivery to plant cells. However, as mentioned earlier, the presence of the ubiquitous plant cell wall as a barrier often complicates application of nanoparticle delivery systems developed for mammalian cells and tissues. Notwithstanding these obstacles, a recent report on the use of functionalized mesoporous nanoparticles (MSNs) for gene transfer to plant cells appears promising (Torney et al. 2007). In this approach, MSNs were filled with β-estradiol, which is an estrogen-receptor-based transactivator (Zuo et al. 2000), for induction of GFP expression, and capped with gold particles coated with the construct of interest and delivered to tobacco plant cells by particle bombardment (Torney et al. 2007). More recently, the use of a new zeolite-based silicalite nanoparticle system consisting of polyethylene imine-plasmid DNA complex was shown to enhance transfection efficiencies in human embryonic kidney cells (Pearce et al. 2008). Zeolite is a crystalline aluminosilicate of open three-dimensional structure with numerous cavities and channels (Mumpton 1999). The inert nature and holding capacity of zeolite therefore opens up new possibilities for gene delivery approaches to plant cells.

3.3.3 Microinjection

One of the most direct methods for gene transfer is to inject DNA directly into the nucleus. Electromechanical devices are used to control the insertion of fine glass

needles into the nucleus of individual cells to deliver the DNA (Spangenberg et al. 1986). Recipient cells or protoplasts are immobilized onto a holding capillary, or in gels, or onto surfaces coated with poly-L-lysine. The nucleus is visualized and the DNA is mechanically injected into it. The recipient cells are cultured and subsequently selected for integration of inserted genes by growth on culture media containing a selective agent. Microinjection has been used to insert genes into individual cells (Nomura and Komamine 1986), microspore-derived embryoids (Neuhaus et al. 1987), or single protoplasts (Steinbiss and Stabel 1983; Morikawa et al. 1986a; Reich et al. 1986). Microinjection method has been used to produce transgenic alfalfa (Reich et al. 1986) by injecting Ti plasmid into alfalfa protoplasts. The microinjection technique is labor intensive and slow, but transformation efficiencies close to 26% were achieved for alfalfa protoplasts (Reich et al. 1986).

3.3.4 Silicon Carbide Whiskers

Silicon carbide is intrinsically very hard and breaks readily to give sharp edges, which are adequate to penetrate plant cell walls. The pores created by the whiskers allow the DNA to be delivered into plant cells. Silicon carbide whiskers are mixed with recipient cells and plasmid DNA by vigorous vortexing and then plated on culture medium. The cultured cells are subsequently assessed for DNA insertion into the cells and integration with nuclear DNA. The silicon carbide whisker-mediated DNA delivery has been shown to produce stably transformed plant cells (Kaeppler et al. 1992, 1994; Kaeppler and Somers 1994) and algae (Dunahay 1993). Silicon carbide whiskers were the most effective in delivering vector DNA as compared to other materials such as silicon nitride or carborundum – a spherical form of silicon carbide and glass beads (Wang et al. 1995). The efficiency of DNA delivery by silicon carbide whiskers could be increased by exposing cells to high molarity of sorbitol or mannitol (Wang et al. 1995). Silicon carbide whiskers have been used to produce transgenic plants of *Lolium*, *Festuca*, and *Agrostis* sp. (Dalton et al. 1998), rice (Matsushita et al. 1999), and maize (Petolino et al. 2000). More recently, a supersonic treatment was combined with the silicon carbide treatment of rice cell suspension cultures, and it was claimed that high efficiency transformation could be obtained (Terakawa et al. 2005).

Although the attributes of silicon carbide whiskers for being simple, cost effective, and less resource demanding for DNA delivery into plants cells have been well recognized, it has also been reported that silicon carbide whiskers exhibit toxic properties and may be harmful to human beings, if not handled with caution (Vaughan et al. 1991; Svensson et al. 1997). There has, therefore, been a search for other possible materials, which could be used for similar DNA transfer purposes. Thus, the use of aluminum borate whiskers (ABW) was suggested as a possible alternative, with previously unreported mutagenic effects and was used to transform scutellar tissues from mature embryos of *Japonica* rice and produced Southern positive transgenic plants (Takahashi et al. 2000). Recent improvements

to the ABW method included use of a multidirectional shaker instead of a vortex and the type of ABW for rice callus transformation (Mizuno et al. 2004). Transgenic tobacco plants have also been produced using the ABW method (Mizuno et al. 2005).

3.3.5 Microprojectile Bombardment

The microprojectile method of DNA delivery, called biolistic (biological ballistic), was invented by Sanford et al. in 1987 as a means of bypassing many of the host range limitations of *Agrobacterium* and also overcoming the physical barrier for Z-DNA uptake by the plant cell wall (Weissinger et al. 1987; Klein et al. 1988b, c). The basic principle underlying microprojectile bombardment is to accelerate microparticles to a speed at which they can penetrate the plant cell wall and be incorporated into the interior of a cell (Sanford 1990). This technique can be used to deliver a range of compounds into a cell and has found applications in several disciplines from agriculture to medicine. Microprojectile bombardment has been used to produce transgenic plants in species which were not amenable to *Agrobacterium*-mediated genetic transformation.

The particle bombardment apparatus essentially consists of a mechanism to accelerate the particles to desired velocities and regulate their penetration into the recipient cells. The original apparatus designed by the inventors used a gun powder discharge to accelerate inert metal microprojectiles coated with biologically active compounds (Sanford 1988; Klein et al. 1988b). The gun powder was quickly replaced with the inert gas, helium (Sanford et al. 1991), to provide the force for microprojection. The main component of the most commonly used helium gun (Biolistics® PDS-1000/He, BioRad, Inc.) is a rupture disk assembly that controls the helium pressure at which the microprojectiles carrying the vector DNA are propelled. The rupture disk assembly consists of a gas acceleration tube with a rupture disk placed at the bottom of the tube inside a retaining cap. The microprojectiles coated with the biological compound of interest are placed on a carrier situated below the rupture disk. The chamber is partially evacuated and the helium gas pressure is allowed to build up to the desired level to rupture the rupture disk. The optimized helium gas pressure propels the microprojectiles at the optimized velocity through a metal screen to deliver biologically active compounds into the target cells. The main consideration in microprojectile-mediated delivery is to deposit in a cell optimal amounts of biologically active compounds with minimal damage to the cell wall. A major limitation of the microprojectile projection technique has been the uneven penetration and distribution of the microprojectiles.

To regulate microprojectile penetration into cells, several modifications have been made to the power source used to propel the microprojectiles carrying the biologically active compounds. Regulated nitrogen gas pressure (Morikawa et al. 1989), compressed air (Iida et al. 1990), or an air gun (Oard et al. 1990) has been used to propel microprojectiles to deliver biologically active compounds into

plant cells. In another approach, a particle in-flow gun (PIG) in which microprojectiles are accelerated directly into a stream of helium rather than being supported by a macrocarrier has been also developed (Finer et al. 1992). An air gun generating a pressure pulse of approximately 2 ms at a pressure of 60 bars to deliver the microprojectile/DNA suspension into the cells has also been used (Sautter et al. 1991). A major difference in this technique is that the DNA is suspended with the microprojectiles rather than being coated on them. The main function of the microprojectiles is to create holes through which DNA passes into the cells. The inventors consider that the movement of DNA independent of microprojectiles allows them to target small areas of tissues. In another modification, electric-discharge-generated shock waves have been used to propel gold microprojectiles coated with DNA or other biologically active compounds (Christou et al. 1988).

Several factors influence the biolistics-mediated delivery of DNA into plant cells. The main determinant is the delivery of optimal amount of DNA with minimal injury to the recipient plant cell or tissue. Material and size of microprojectiles, attachment of DNA to microprojectiles, pressure at which microprojectiles are propelled and the recipient tissue are all critical factors. Most of these parameters need to be optimized and vary with individual laboratories. By only adjusting the distance that the microparticles travel to the target tissue or changing the target diameter, it can be shown that transformation events may be affected. Using GUS histochemical assays it can be shown that the number of GUS-positive spots was greatly increased when the flight distance of the microparticles was set at six centimeters and the target diameter of one centimeter, consisting in this example of wheat mature embryos tightly packed within this area (Fig. 3.3). Increasing the flight distance and the diameter of the target area led to fewer GUS spots (Fig. 3.3). Thus depending on the type of tissues, parameters for particle bombardment can be

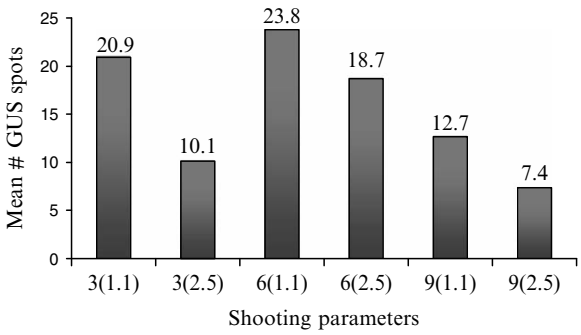

Fig. 3.3 Distance between target tissue and macrocarrier holder containing DNA-coated gold microparticles and their influence on the number of GUS-positive spots on wheat mature embryos. Numbers on x axis indicate target distance in cm and number in parentheses indicate target diameter in cm

accordingly optimized for maximal transient expression, prior to performing actual transformation experiments. Transient expression based on the *Gus* gene or the *GFP* gene is a good indication of adequate coverage of the tissue in terms of possible integration of the transgene across the tissue surface. Biolistics-mediated transformation has been used to genetically transform a large number of plant species including major agricultural crops such as maize, cotton, soybeans, rice, sorghum, and wheat and is likely to be the method of choice for plant species where *Agrobacterium*-mediated transformation is still inefficient.

3.3.6 Electroporation

Short, high-intensity electric pulses reversibly permeabilize the lipid bilayers of cell membranes in all living cells. The electric pulses cause extensive compression and thinning of the plasmalemma, resulting in the transient formation of pores in the plasma membrane (Neumann and Rosenhec 1972). The transiently formed pores allow the diffusion of a range of macromolecules including proteins and nucleic acids. The apparatus used for electroporation is fairly simple. High electric fields are applied to protoplasts, cells, or tissues suspended in a liquid culture medium enclosed in a discharge chamber. The electric field is applied by a capacitor discharge which in some commercial instruments can produce voltage up to 2,500 V. The time and voltage applied depends upon the cell and tissue type used for electroporation.

Electroporation has been used to deliver DNA in a range of plant cells and tissues. However, protoplasts, which lack cell walls, are the most amenable recipient system for electroporation-mediated DNA delivery. Electroporation-mediated gene transfer into plant protoplasts has been shown in several plants such as tobacco (Shillito et al. 1985; Negrutiu et al. 1987), corn (Fromm et al. 1986), rice (Shimamoto et al. 1989), soybean (Dhir et al. 1991), sugarcane (Chowdhury and Vasil 1992), and oilseed rape (Bergman and Glimelius 1993). Even though over the years there have been many other reports of transformation of protoplasts from other plant species by electroporation, the major limitation has been regeneration of fertile transgenic plants from the electroporated protoplasts for a number of species. This was even more problematic for monocotyledonous species. There was, therefore, an interest in bypassing the protoplasts for electroporation. It was reported that tobacco pollen grains subjected to electroporation treatments stayed viable (Mishra et al. 1987) and that electroporation of barley microspores with propidium iodide subsequently produced callus that regenerated plants (Joersbo et al. 1990). Successful DNA delivery into tobacco pollens by electroporation was also reported, including transient GUS expression and positive Southern hybridization signals from blots with DNA extracted from the transformed pollen grains (Abdulbaki et al. 1990; Matthews et al. 1990). Since then there have been numerous reports on optimizing DNA delivery into intact plant cells or tissues, mainly pertaining to the duration of electric pulses and field strength. The first report of stable

transformation via electroporation of intact tissues was with rice seeds cultured 2 days prior to being subjected to electroporation with a plasmid containing *NPTII* gene (Li et al. 1991). Subsequently, electroporation of immature corn embryos and callus (D'Halluin et al. 1992) and nodal buds of pea and lentils (Chowrira et al. 1995) led to the production of stable transformants.

3.3.7 Other Potential Physical Methods

Over the years that followed physical methods of transformation of plants by electroporation and biolistic, attempts at developing other versatile and less expensive methods have been made, including improvising existing systems (Table 3.1). Among these developments, some of the approaches based on alginate microbeads in combination with PEG and/or electroporation or DNA-lipofectin complexes (Sone et al. 2002; Liu et al. 2004; Murakawa et al. 2008a, b) are promising. However, only the method using PEG for uptake of the bioactive beads was shown to produce transgenic plants that were Southern positive. Thus, the requirement for protoplasts would still be a major impediment for the bioactive beads-based transformation system due to difficulties still associated with regeneration of fertile plants from protoplasts for a vast majority of plant species.

3.4 Combined Physical and Biological DNA Delivery

3.4.1 Agrolistic

Attempts to maximize the benefits of the different available gene transfer methods in plants have been extensively pursued and are still actively being explored. It has been widely recognized that both the *Agrobacterium*-mediated and the biolistic-mediated gene transfer approaches have their respective advantages and disadvantages, depending on the species and circumstantial requirements. Thus, to utilize the benefits of these two systems, a combination of *Agrobacterium*-mediated transformation with the biolistic delivery was developed and was termed the "agrolistic" transformation system (Hansen and Chilton 1996). In this system, the *virD1*/*virD2* genes are cotransformed by particle bombardment with a plasmid DNA containing the T-DNA borders flanking the gene of interest. Thus, there is transient expression of the virD1/D2 proteins and excision of the T-DNA occurs in planta similar to that with *Agrobacterium*-mediated transformation. Even though this method has included the underlying benefits of both systems, reports on the successful use of this approach for the generation of stably transformed plants have been lacking.

3.4.2 Sonication-Assisted Delivery

Further improvement to the *Agrobacterium* transformation method included brief treatments of the plant tissues with ultrasound in the presence of *Agrobacterium*, a method called sonication-assisted *Agrobacterium*-mediated transformation (SAAT) (Trick and Finer 1997). Sonication as such has previously been used in conjunction with gene transfer to protoplasts of *Beta vulgaris* and tobacco (Joersbo and Brunstedt 1990). Using the sonication approach, leaf segments of tobacco showed transient expression and regenerated R_1 plants exhibited stable expression of the transgenes (Zhang et al. 1991). Unlike the agrolistic method there have been several reports on the successful production of stably transformed plants using the SAAT method (Christiansen et al. 2000; Pathak and Hamzah 2008; Rashid et al. 2008). It is likely that further refinement using the SAAT method will lead to more reports on the stable transformation of other plant species.

3.5 Concluding Remarks

Development of gene transfer methods in plants has probably been one of the most challenging aspects of plant research. Currently the two methods of choice are undoubtedly the *Agrobacterium*-mediated and the biolistic-mediated DNA delivery systems. In the latter two decades of the last century, development of transformation technology was viewed primarily as an objective to the production of transgenic crops with improved agronomic characteristics for enhanced crop productivity. However, the emergence of functional genomics and the requirement for high-throughput technology for assessment of gene function in plants have generated a whole new focus on generation of transgenic plants. This has become even more evident for economically important crop plants, wherein high efficiency transformation systems are generally lacking, compared to model systems such as *Arabidopsis thaliana*. Nonetheless progress made in rice and corn is encouraging with the use of *Agrobacterium* for transformation in a fairly high-throughput manner. The focus is, therefore, still centered around those other economically important plants, which can be made amenable for high-throughput gene transfer in order to make utmost use of the vast repertoire of data flowing from genome projects.

Thus, gene transfer methods to plants will continue to receive renewed interest in the future. The development of nanoparticles for DNA delivery into plant cells is emerging as the next generation transformation system and is likely to combine the benefits of *Agrobacterium* and biolistic. Meanwhile, the approach of in planta transformation using *Agrobacterium* (Bechtold et al. 1993; Clough and Bent 1998) is likely to continue receiving attention, due to circumvention of a tissue culture step for the regeneration of transformation events. In planta transformation for several species such as *Brassica rapa* ssp. *chinensis* (Qing et al. 2000),

Medicago truncatula (Trieu et al. 2000), *Hibiscus canabinus* (Kojima et al. 2004), rice (Supartana et al. 2005), and wheat (Supartana et al. 2006) has been reported. However the transformation efficiency achieved by this method thus far for all these species is yet to be convincing and requires further refinement and research. Furthermore, several of the other different methods reviewed in this chapter and listed in Table 3.1 are likely to be explored further. The likely successes, however, will come from a combination or refinement of several existing methods, with the most suitable, and most likely popular, gene transfer method possessing the versatility, simplicity, and accessibility for a large number of plant species.

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Chapter 4
Selection and Screening Strategies

Haiying Liang, P. Ananda Kumar, Vikrant Nain, William A. Powell, and John E. Carlson

4.1 Introduction

A number of transformation systems have been developed to insert foreign DNA into the appropriate plant genome (nuclear or plastid) (discussed in Chap. 3). However, only a small fraction of the treated cells become transgenic, while the majority of the cells remain untransformed using any of these methods. Thus, effective selection and screening strategies are needed to pick up the rare transgenic lines from a pool of nontransformed cells or plants. To date, more than 50 marker genes and a few molecular techniques have been developed to serve this essential purpose.

In general, a marker gene is cointroduced into a plant genome along with the transgene of interest to help identify the cells that have taken up the foreign DNA, which is especially important when the transformation frequency is low (e.g., 1.0×10^{-3} to 10^{-6}) (Curtis et al. 1995). In some cases, the marker gene is the gene of interest that will express an agronomic characteristic, such as herbicide resistance. Marker genes can be divided into two categories, i.e., selectable markers and screenable (scorable, reporter, visible) markers. Selectable markers are genes that can provide a selective/metabolic advantage to the transformed cells for them to grow under conditions, which inhibit the growth of nontransformed cells. Selectable markers allow selective multiplication of transformed cells by killing or starving the nontransformed ones. Examples of selectable markers include genes that provide either antibiotic or herbicide resistance, or necessary growth regulators. Screenable markers usually encode for specific proteins, such as β-glucuronidase (GUS), green fluorescent protein (GFP), and luciferase (LUX), that can produce distinctive phenotypes, thus enable the identification of transformed cells without adding any toxic compounds. Assays for screenable markers can be destructive or nondestructive. Screenable markers are helpful in monitoring transgenic events and manually

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separating transgenic tissues from nontransformed tissues, but provide no selection pressure on cells or regenerated shoots. It is not a surprise that identifying transgenic cells among many more nontransgenic cells only with screenable markers can be time consuming. Therefore, screenable markers are usually paired with selectable markers in transformation systems. Indeed, studies have shown that the number of transgenic events is reduced almost ten-fold in the absence of selectable markers (Birch 1997; de Vetten et al. 2003; Darbani et al. 2007).

Here we describe the characteristics of individual selectable and screenable marker genes available to date for plant transformation and their applications in production of transgenic plants. The molecular tools that can be utilized for transformant screening and the strategies for marker gene removal after successful transformation events are also described.

4.2 Selection Strategies

Selection strategies can be classified into two categories depending on whether they confer an advantage (positive) or a disadvantage (negative) selection. In negative selection, a more traditional mode of selection, toxic or inhibitory compounds such as antibiotics or herbicides are used to kill or prevent the growth of nontransformed cells. In the case of positive selection, transformed cells are given the ability to grow by using a specific carbon or nitrogen source or a growth regulator as the selection agent. Thus, positive selectable marker genes are defined as those that promote the growth of transformed tissue, whereas negative selectable marker genes result in death or growth inhibition of the nontransformed tissue. As a relatively new mode of selection strategy, positive selection has been demonstrated to be successful in a large variety of monocot and dicot species, and usually provides a higher transformation frequency than negative selection.

4.2.1 Positive Selection

4.2.1.1 Shoot/Root Phenotypic-Based Positive Selection (Table 4.1)

Isopentyl Transferase

The enzyme isopentyl transferase (IPT) contributes to crown gall formation in infected plants and is encoded by the T-DNA of *Agrobacterium tumefaciens* Ti plasmids. IPT catalyzes the first step in cytokinin biosynthesis, the synthesis of isopentyl-adenosine-5′-monophosphate, leading to elevated cytokinin levels in transgenic plants. Since high cytokinin:auxin ratios are required for shoot formation in culture, introduction of the *ipt* gene into plants can enhance regeneration of shoots without the inclusion of exogenous cytokinin in the media. It has been

Table 4.1 Agents and enzymes for shoot/root phenotypic-based positive selection

Agents	*Genes*	Enzymes	Sources	Genome	References
None	*ipt*	Isopentyl transferases	*Agrobacterium tumefaciens*	Nuclear	Endo et al. (2001)
	pga22		*Arabidopsis thaliana*		Zuo et al. (2002)
None	*esr1*	Transcription factor (enhancer of shoot regeneration 1)	*Arabidopsis thaliana*	Nuclear	Banno et al. (2001)
None	*cki1*	Histidine kinase (cytokinin-independent 1)	*Arabidopsis thaliana*	Nuclear	Zuo et al. (2002)
None	*knotted1*	Transcription factor (enhancer of shoot regeneration 1)	*Zea mays*	Nuclear	Luo et al. (2006)
Benzyladenine-N-3-glucuronide	*uidA* (*gusA*)	β-Glucuronidase	*Escherichia coli*	Nuclear	Joersbo and Okkels (1996)
None	*rol*	Enzymes involved in rhizogenesis	*Agrobacterium rhizogenes*	Nuclear	Ebinuma et al. (1997)

demonstrated that the *ipt* gene is effective as a positive selectable marker for transformation in tobacco, tomato, muskmelon, sweet pepper, and citrus (Kunkel et al. 1999; Endo et al. 2001, 2002; Mihálka et al. 2003; Ballester et al. 2007). However, the main issue with this system is that transgenic plants usually show abnormal phenotypes, such as loss of apical dominance and roots, when *ipt* is expressed constitutively. Therefore, the *ipt* gene cannot be constitutively expressed in the same way as traditional markers. Two approaches have been used to avoid this drawback. In one approach, the marker has been excised from the transgenic plant by inducible site-specific recombination (Sugita et al. 2000). The other approach has been to place the *ipt* gene under an inducible promoter, so that cytokinins are produced only when needed. Thus, regeneration is carried out in cytokinin-free medium in the presence of the inducing agent, so that only transformed cells produce the enzyme required to stimulate cytokinin synthesis (Zuo et al. 2002).

Plant *ipt* genes have been cloned from *Arabidopsis* and investigated as possible selectable markers (Kakimoto 2001; Takei et al. 2001). Overexpression of *Arabidopsis* IPT (e.g., *PGA22*) has been found to promote shoot formation from explants in the absence of external cytokinins. Direct selection with plant *ipt* genes needs to be tested in crop species.

Enhancer of Shoot Regeneration 1

The *Arabidopsis* enhancer of shoot regeneration 1 (*ESR1*) gene encodes an AP2/EREBP (APETALA2/ethylene response element binding protein)-domain-containing

transcription factor. It can cause high-frequency shoot regeneration in the absence of external cytokinins (Banno et al. 2001). Several *ESR1*-like genes, e.g., *ESR2*, were also found in the *Arabidopsis* genome. The utility of the *ESR1* and *ESR1*-like genes as promising markers in genetic transformation of crop plants has not been demonstrated.

Histidine Kinase

The *Arabidopsis Cytokinin-Independent 1* (*CKI1*) gene appears to be a receptor-like histidine kinase, and was proposed as the putative cytokinin receptor (Kakimoto 1996). Overexpression of *CKI1* was able to promote shoot regeneration independent of exogenous cytokinins and caused typical cytokinin responses. Under the control of the β-estradiol-inducible promoter and in the presence of the inducer β-estradiol, *CKI1* served as a successful selection marker in *Arabidopsis* and tobacco in the absence of external cytokinins. All transformed shoots were found to develop into normal adult plants when transferred onto a noninductive medium and no nontransgenic escapes were found among the regenerated plants (Zuo et al. 2002).

Homeodomain-Containing Knotted1 Protein

Homeobox gene *knotted1* (*kn1*) is normally expressed in shoot meristem and appears to play a critical role in meristem initiation (Hake et al. 2004). Transgenic plants overexpressing *kn1* gene exhibit morphological alterations that are similar to the characteristics of *ipt*-expressing plants, including changes in leaf shape, loss of apical dominance, and production of ectopic meristems on leaves. Although the functional mechanism of the *kn1* gene is not well understood, the maize *kn1* gene produced similar effects in selection of transgenic plants as the *ipt* gene in the absence of cytokinin and auxin (Luo et al. 2006). Under the control of the cauliflower mosaic virus (CaMV) 35S promoter, transformation efficiencies with the maize *kn1* gene as selectable marker were slightly higher than those with the *ipt* gene, while three-fold higher than neomycin phosphotransferase II (*nptII*) gene. Like *ipt* gene, abnormal morphology is a major drawback of using *kn1* as a selectable marker.

β-Glucuronidase

Benzyladenine *N*-3-glucuronide is an inactive, glucuronide derivative of cytokinin. When hydrolyzed by β-glucuronidase (GUS, E.C. 3.2.1.31), benzyladenine is released from benzyladenine *N*-3-glucuronide and stimulates transformed cells to regenerate. Joersbo and Okkels (1996) first applied the *E. coli gusA* gene as a positive selection strategy in tobacco. When paired with 7.5–15 mg L^{-1}

benzyladenine *N*-3-glucuronide, the transformation frequency scored by shoot regeneration was 1.7–2.9-fold higher than that achieved by the *nptII* gene in control experiments. In addition, *gusA* can be used as a scorable marker, which obviates the need for any traditional selectable marker gene.

Root Locus (ROL) Proteins

The *root locus (ROL)* genes from the T-DNA of *Agrobacterium rhizogenes* Ri-(root-inducing) plasmid have profound effects on root development. Vilaine and Casse-Delbart (1987) and Schmulling et al. (1988) reported that a cluster of *rolA*, *rolB*, and *rolC* genes (*rolABC*) is sufficient to induce a typical root proliferation response (hairy root) on *A. rhizogenes*-infected plants in vitro when no exogenous auxins are supplied to the cultivation medium. *Rol* genes have provided a hairy root phenotypic-based selection scheme in transgenic tobacco and *Antirrhinum snapdragon* (Cui et al. 2001; Komarnytsky et al. 2004). Hairy roots are not desirable in standard transformation. Thus, *rol* genes usually are deleted after transformation and selection are done.

4.2.1.2 Carbon-Based Positive Selection (Table 4.2)

Xylose Isomerase

Plant cells from species such as potato, tobacco, tomato, and several coffee species cannot use D-xylose as a sole carbon source, unless D-xylose is isomerized to D-xylulose by xylose isomerase (D-xylose ketol-isomerase, E.C. 5.3.1.5). Xylose isomerase/xylose selection was efficient in these plants (Haldrup et al. 2001; Samson et al. 2004). The xylose isomerase genes (*xyl*A) employed were from *Thermoanaerobacterium thermosulfurogenes* or *Streptomyces rubiginosus*.

Table 4.2 Agents and enzymes for carbon-based positive selection

Agents	Genes	Enzymes	Sources	Genome	References
D-Xylose	*xylA*	Xylose isomerase	*Streptomyces rubignosus,* Thermoanaerobacterium thermosulfurogenes	Nuclear	Haldrup et al. (1998)
D-Mannose	*manA (pmi)*	Phosphomannose isomerase	*Escherichia coli*	Nuclear	Joersbo and Okkels (1996)
D-Arabitol	*atlD*	d-Arabitol dehydrogenase	*Escherichia coli*	Nuclear	LaFayette et al. (2005)

Phosphomannose Isomerase

Mannose is not toxic to plant cells. However, plant cells can take up mannose and use hexokinase to convert it to mannose-6-phosphate, an inhibitor of glycolysis. The production of mannose-6 phosphate also depletes the cell of inorganic phosphate. Phosphomannose isomerase (PMI, E.C. 5.3.1.8) converts mannose-6-phosphate to fructose-6-phosphate, an intermediate of glycolysis, thus allows mannose to become a carbon source. Phosphomannose isomerase is absent in many plants except leguminous plants (Lee and Matheson 1984; Chiang and Kiang 1988). Using mannose as the selective agent (usually in combination with sucrose or glucose), the *E. coli manA* (*pmi*) gene under the control of the CaMV 35S promoter was found to be an effective selectable marker. Since its first demonstration in potato, sugar beet, and corn in 1999 (Bojsen et al. 1999), the *pmi*/mannose selection system has been utilized in many crop species, including rice, sweet orange, wheat, papaya, barley, watermelon, tobacco, sorghum**,** Chinese cabbage, cucumber, almond, apple, and sugarcane (Reed et al. 2001; Sigareva et al. 2004; Gao et al. 2005; Zhu et al. 2005; Degenhardt et al. 2006; He et al. 2006; Ramesh et al. 2006; Jain et al. 2007; Min et al. 2007; and references therein). In most of the cases, transformation frequencies obtained from the *pmi*/mannose selection scheme were higher than antibiotics selection, and no adverse phenotypes were observed. Besides legumes, which have PMI activity, this *pmi* system is also not suitable for transformation of grapevine and coffee since grapevine and coffee embryos can use mannose or xylose as the sole carbohydrate source (Kieffer et al. 2004; Samson et al. 2004).

D-Arabitol Dehydrogenase

While many plants cannot metabolize most sugar alcohols, including D-arabitol (Stein et al. 1997), some bacteria contain an arabitol gene encoding D-arabitol dehydrogenase (EC 1.1.1.11). This enzyme converts arabitol into xylulose on which plants can grow, since xylulose is an intermediate of the oxidative pentose phosphate pathway (Haldrup et al. 1998). An *E. coli* form of D-arabitol dehydrogenase encoding gene, *atlD,* was plant-codon modified and expressed in rice under the control of a CaMV 35S promoter. Selection with 27.5 g L^{-1} arabitol and 2.5 g L^{-1} sucrose resulted in transformation rate that was comparable to selection with hygromycin and *pmi*, while transgenic rice plants obtained with the arabitol selection scheme appeared morphologically normal during differentiation and regeneration (LaFayette et al. 2005).

4.2.1.3 Auxotrophic Markers

Auxotrophic mutants can be supplemented by transformation with a functional gene, which can serve as a selectable marker gene. Such auxotrophic marker gene

enables transformed cells to synthesize an essential component, usually an amino acid, which the cells cannot otherwise produce. The surrounding medium is made to intentionally lack the essential component, which cells require to grow. Cells that have successfully incorporated the selectable marker and the rest of the gene construct will produce the essential components within the cells, and thereby survive. While this technology is commonly used in bacteria and yeast transformation, it is not widely utilized in plants due to the lack of homozygous mutants that require nutritional supplements (Aragão and Brasileiro 2002). Examples include two tobacco mutants that are deficient in threonine dehydratase or nitrate reductase (Vincentz and Caboche 1991).

4.2.1.4 Selection with Biotic and Abiotic Stresses (Table 4.3)

Pathogen Resistance

A sweet pepper ferredoxin-like protein (pflp) has antimicrobial activity in planta (Lin et al. 1997; Tang et al. 2001; Liau et al. 2003). You et al. (2003) and Chan et al. (2005) successfully utilized the *pflp* gene as selection marker and a bacterial pathogen *Erwinia carotovora* as the selection agent for transformation of *Oncidium* orchid. This selection scheme has not been reported in crops.

High Salt Tolerance

Coupled with 200 mM NaCl as the selectable agent, two salt tolerance genes *DREB2A* and *SOS1*, cloned from rice and *Arabidopsis*, respectively, were superior to using an antibiotic or herbicide for selection in producing salt tolerant rice plants (Zhu and Wu 2008).

Heat Shock Tolerance

Overexpression of the plant heat shock protein gene *HSP101* confers basal thermotolerance in crops such tobacco (Chang et al. 2007) and rice (Katiyar-Agarwal et al.

Table 4.3 Agents and enzymes for biotic and abiotic stress-based positive selection

Agents	Genes	Enzymes	Sources	Genome	References
Erwinia carotovora (Baterial pathogen)	*pflp*	Ferredoxin-like protein	*Capsicum annuum*	Nuclear	You et al. (2003), Chan et al. (2005)
200 mM NaCl	*dreb2a*	Transcription factor	*Oryza sativa*	Nuclear	Zhu and Wu (2008)
	sos1	Plasma membrane antiporter	*Arabidopsis thaliana*		
47°C, 60 min	*Hsp101*	Heat shock protein	*Oryza sativa*	Nuclear	Chang et al. (2007)

Table 4.4 Agents and enzymes for antibiotics-based positive selection

Agents	Genes	Enzymes	Sources	Genome	References
Spectinomycin, Streptomycin	*aadA*	Aminoglycoside-3‴ adenyl transferase	*Shigella* sp.	Nuclear Plastid	Svab et al. (1990) Svab and Maliga (1993)
Streptomycin	*spt*	Streptomycin phosphotransferase	Tn5	Nuclear	Maliga et al. (1988)

2003). The feasibility of using the rice heat shock protein gene (*osHsp101*) as a selection marker was successfully demonstrated in rice under heat treatment (47°C, 60 min) for selection (Chang et al. 2007).

4.2.1.5 Antibiotics-Based Positive Selection

Under appropriate conditions, some antibiotics like streptomycin and spectinomycin bleach sensitive plant cells instead of killing them, while resistant plants stay green, thus provide a color differentiation between wild-type and transgenic plants (Table 4.4). The streptomycin phosphotransferase (*SPT*) gene from Tn5 provides resistance to streptomycin and has been used to select transgenic tobacco, driven by the T-DNA transcript 2′ promoter (Maliga et al. 1988). The efficiency of transformation using this streptomycin resistance marker was found comparable to the *npt*II gene under the control of the nopaline synthase (*nos*) promoter (Maliga et al. 1988). The *SPT* marker was also successfully applied to monitor transposon excision by providing a cell autonomous resistance phenotype (Ziemienowicz 2001). However, this marker system has not been adopted for general use. The bacterial aminoglycoside-3‴-adenyl-transferase gene *(aad*A*)* conferring resistance to both streptomycin and spectinomycin (Svab et al. 1990) has been used as a selectable marker in tobacco, white clover, and maize (Miki and McHugh 2004). While this gene has not been broadly utilized as a nuclear selectable marker gene for the production of transgenic plants, it is the most widely used selectable marker for plastid transformation.

4.2.2 Negative Selection

4.2.2.1 Antibiotics (Table 4.5)

Neomycin Phosphotransferase

Neomycin phosphotransferase (also known as aminoglycoside3′-phosphotransferase) confers resistance to various aminoglycoside antibiotics, including kanamycin,

Table 4.5 Toxic antibiotics for negative selection

Antibiotics	Genes	Enzymes	Sources	Genome	References
Neomycin	*neo, nptII*	Neomycin phosphotransferase	*Escherichia coli Tn5*	Nuclear	Fraley et al. (1983)
Kanamycin				Plastid	Carrer et al. (1993)
Paramomycin, G418					
Hygromycin B	*hph* (*aphIV*)	Hygromycin phosphotransferase	*Escherichia coli*	Nuclear	Waldron et al. (1985)
Aminoglycosides	*aaC3*	Aminoglycoside-N-acetyl transferases	*Serratia marcesens*	Nuclear	Hayford et al. (1988)
	6´ gat		*Shigella sp.*		Gossele et al. (1994)
Bleomycin	*Ble*	Bleomycin resistance	*Escherichia coli Tn5* *Streptoalloteichus*	Nuclear	Hille et al. (1986) Perez et al. (1989)
Sulfonamides	*sulI*	Dihydropteroate synthase	*Escherichia coli pR46*	Nuclear	Guerineau et al. (1990)
Streptothricin	*sat3*	Acetyl transferase	*Streptomyces sp*	Nuclear	Jelenska et al. (2000)
Chloramphenicol	*cat*	Chloramphenicol acetyl transferase	*Escherichia coli Tn9*	Nuclear	DeBlock et al. (1984)
			Phage p1cm	Plastid	DeBlock et al. (1985)
Kanamycin	*Atwbc19*	ATP binding cassette (ABC) transporter	*Arabidopsis thaliana*	Nuclear	Mentewab and Stewart (2005)

neomycin, geneticin (G418), butirosin, gentamycin B, and paromomycin (Norelli and Aldwinckle 1993), by catalyzing the transfer of the terminal phosphate of ATP to the drug (Jimenez and Davies 1980). In plants, because of this ATP-dependent phosphorylation, binding of the antibiotic to ribosomes in mitochondria and chloroplasts is prevented; thus, protein synthesis is not impaired. Three kinds (I, II, and III) of neomycin phosphotransferase genes (*npt* or *neo*) have been used as selection markers in plants. Among them, *nptII* (neomycin phosphotransferase II, E.C 2.7.1.95) gene from *E. coli* transposon has become the most widely used since it was first established in 1983 (Bevans et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983). Many crop plants, such as maize, cotton, tobacco, soybean, almond, and poplar, have been successfully transformed with the *nptII* gene. Endogenous NPTII activity is very rare in plant tissues. No adverse effects of either NPTII enzyme or the *nptII* gene on humans, animals, or the environment have been reported (Flavell et al. 1992; Nap et al. 1992; US Food and Drug Administration 1998; European Food Safety Authority 2007). In addition, NPTII protein activity can be detected by enzymatic assay.

Hygromycin Phosphotransferase

The hygromycin phosphotransferase (HPT, HPH, E.C. 2.7.1.119) enzyme, also known as aminoglycoside 4′-phosphotransferase (APHIV), gives resistance to

hygromycin B antibiotic (Van Den Elzen et al. 1985). Like neomycin phosphotransferase, HPT catalyzes the phosphorylation of the hydroxyl group in the hygromycin antibiotic thus preventing its binding to ribosomes. The *hpt* gene was originally derived from *E. coli* and has been extensively utilized, especially when the use of the *neo* gene is not possible. Hygromycin B is also an aminoglycoside antibiotic, causing the same symptoms as other aminoglycoside antibiotics (Benveniste and Davies 1973). As the second most frequently used antibiotic for selection after kanamycin, hygromycin B has proved very effective in the selection of a wide range of plants, including monocots. Compared to kanamycin, hygromycin B is usually more toxic and kills sensitive cells more quickly. Since hygromycin B exhibits highly toxic effects in mammalian cells, careful working procedures are recommended (McDaniel and Schultz 1993). Working concentrations range from 20 to 200 mg ml^{-1} for plant cells.

Aminoglycoside-3-N-acetyltransferase (ACC3) and Aminoglycoside-6-N-acetyltransferase (ACC6)

The aminoglycoside acetyltransferases comprise four classes of enzymes, designated AAC(1), AAC(2′), AAC(3), and AAC(6′), according to the site of acetylation of the deoxystreptamine core of the aminoglycoside antibiotic (Braeu et al. 1984). These enzymes are common among both gram-negative and gram-positive bacteria. Three genes encoding the AAC(3) enzyme, *acc*(3)-I, *acc*(3)-III, *acc*(3)-IV, have been used successfully as selectable markers for transformation of canola, tobacco, and tomato (Hayford et al. 1988). The enzymes AAC(3)-III and AAC(3)-IV have broad substrate specificity, detoxifying gentamycin, kanamycin, tobramycin, neomycin, and paromomycin by acetylation. AAC(3)-IV also modifies aparmycin and G418. The ACC(3)-I enzyme, on the contrary, modifies only gentamycin and some close derivatives (i.e., fortimicin), and may be useful if one wants to combine it with other selection markers (Shaw et al. 1993). Lastly, an AAC(6) encoding gene *6′ gat* from *Shigella* spp., when under the control of the CaMV 35S promoter, was proved as efficient for selection of transformed tobacco protoplasts as *nptII* on high levels of kanamycin (Gossele et al. 1994).

Bleomycin-Binding Protein

Bleomycins are a family of metalloglycopeptide antibiotics. They bind to specific DNA sequences and produce single-stranded and double-stranded DNA breaks. An analog of bleomycin, phleomycin differs in that one of the two thiazole ring moieties is partially saturated (Sugiura et al. 1985). Encoding a bleomycin-bind protein, two bleomycin resistance determinants (ble) from *E. coli* transposon Tn5 (*Tn5Ble*) and chromosome of *Streptoalloteichus hindustanus* (*ShBle*), respectively, have been cloned. The binding of Tn5Ble and ShBle proteins to bleomycin is irreversible, thus rendering them inactive. So far *Tn5Ble* and *ShBle* genes have

been introduced into *Nicotiana* (Hille et al. 1986; Perez et al. 1989; El Amrani et al. 2004); however, only selection with phleomycin was able to generate transgenic plants. Several reports have indicated that bleomycin inhibits plant morphogenesis and transgenic plant production and *ShBle* was found to be more effective than Tn5ble under the control of the CaMV 35S promoter (Perez et al. 1989; Singh and Sansavini 1998; Schmidt et al. 2008).

Dihydropteroate Synthase

Dihydropteroate synthase (DHPS, E.C. 2.5.1.15) catalyzes a rate-limiting step for folic acid synthesis in bacteria and plants, and its enzymatic activity can be inhibited by a large number of antimicrobial compounds such as sulfonamides or sulfa drugs. The resistance gene *sul*I from plasmid R46 codes for a mutant form of DHPS that is resistant to inhibition by the sulfonamides. Highly efficient selection systems based on *sul*I and sulfonamides were demonstrated in tobacco (Guerineau et al. 1990) and potato (Wallis et al. 1996). In both cases, the mutant form of dihydropteroate synthase was targeted to the chloroplast.

Streptothricin Acetyltransferase

Streptothricins are antimicrobial agents produced by *Streptomyces* spp. The mechanism of action of streptothricins is similar to that of aminoglycoside antibiotics: inhibition of protein synthesis by binding to the ribosomal small subunit (see for review Jelenska et al. 2000). The *E. coli sat3* gene codes for an acetyl transferase activity that inactivates streptothricins. When controlled by the 35S promoter, the *sat* gene acted as a selectable marker gene in a variety of dicotyledonous plant species including tobacco and carrot (Jelenska et al. 2000).

Chloramphenicol Acetyltransferase

Antibiotic chloramphenicol inhibits protein synthesis and the uptake of cations and anions in higher plants (Jyung et al. 1965). Chloramphenicol acetyltransferase (CAT) (E.C. 2.3.1.2, CAT) catalyzes the transfer of an acetyl group from acetyl-CoA to the 3′-hydroxy position of chloramphenicol, thus inhibiting chloramphenicol from binding to the ribosome. The *cat* gene from *E. coli* Tn*9* or Phage p1cm, driven by the *nos* promoter, has been used for the selection of tobacco transformants by introduction into the nuclear or chloroplast genomes (DeBlock et al. 1984, 1985). However, selection on chloramphenicol was much less efficient than selection on kanamycin conferred by the *nptII* gene. This gene is primarily used as a reporter gene rather than a selectable marker.

ATP-binding Cassette (ABC) Transporter

An *Arabidopsis* ATP-binding cassette (ABC) transporter (*Atwbc19*) gene has been shown recently to confer kanamycin resistance in transgenic tobacco (Mentewab and Stewart 2005). Under the control of the CaMV 35S promoter, the ABC transporter's selection efficiency is comparable to that of *nptII*. This is the first identified plant gene that confers antibiotic resistance. The mechanism of action is not clear. It has been hypothesized that kanamycin is actively sequestered in the vacuole as a substrate of this ABC transporter, where it would not interfere with ribosomes in the cytoplasm, mitochondria, and chloroplasts, thereby mitigating its toxicity (Mentewab and Stewart 2005).

4.2.2.2 Herbicides (Table 4.6)

Phosphinothricin Acetyltransferase

Phosphinothricin (PPT) is an active ingredient in the broad-spectrum herbicide Basta. PPT is an analog of glutamate that inhibits the amino acid biosynthetic enzyme glutamine synthase (GS) of plants and bacteria. In plants, GS is involved in assimilation of ammonia and in regulation of nitrogen metabolism. Inhibition of GS by PPT causes rapid accumulation of intracellular ammonia levels, which leads to disruption of chloroplast structure resulting in inhibition of photosynthesis and plant cell death (Tachibana et al. 1986). PPT resistance genes *bar* and *pat* from *Streptomyces* sp. encode phosphinothricin acetyltransferase (PAT), an enzyme that acetylates the free NH_2 group of PPT, thereby rendering it nontoxic. The PAT enzymes encoded by these two genes are functionally identical and show 85% identity at the amino acid level (Wohlleben et al. 1988; Wehrmann et al. 1996). The *bar* gene is the most widely and successfully used selection marker for all the major cereal species. Basta or PPT can be used to select for PPT-resistant plants by spraying full-grown plants or by adding it to selective medium in earlier stages. In the media, 1–10 mg L^{-1} PPT is adequate to select for transformed cells in many plant species.

5-Enolpyruvyl-Shikimate-3-Phosphate Synthase and Glyphosate Oxidase

The plastid enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, E.C. 2.5.1.19) is essential in the shikimate pathway for the biosynthesis of the aromatic amino acids (e.g., tryptophan, tyrosine, and phenylalanine) in plants and bacteria and a primary target of herbicide glyphosate. EPSP synthase uses phosphoenol pyruvate (PEP) and shikimate-3-phosphate as substrates to make EPSP. However, glyphosate competitively interferes with the binding of PEP to the active site of EPSP synthase, hence blocking the pathway (Anderson et al. 1988; Schönbrunn et al. 2001). Both overexpression of wild-type EPSPS and expression of mutant

Table 4.6 Toxic herbicides and selectable marker genes used for the conditional-positive selection of transgenic plants[a]

Herbicides	Genes	Enzyme	Source	Genome	References
Phosphinothricin	*pat, bar*	Phosphinothricin acetyl transferase	*Streptomyces hygroscopicus, Streptomyces viridochromogenes Tu494*	Nuclear	DeBlock et al. (1989)
Glyphosate	*epsps*	5-Enolpyruvylshikimate-3- phosphate synthase	*Petunia hybrida, Zea may, Oryza sativa*	Nuclear	Zhou et al. (1995), Howe et al. (2002), Charng et al. (2008)
	aroa		*Salmonella typhimurium, Escherichia coli*		Comai et al. (1988), Della-Cioppa et al. (1987)
	cp4 epsps		*Agrobacterium tumefaciens*		Barry et al. (1992)
	gox	Glyphosate oxidoreductase	*Ochrobactrum anthropi*		Barry et al. (1992)
Sulfonylureas	*csr1-1*	Acetolactate synthase	*Arabidopsis thaliana*	Nuclear	Olszewski et al. (1988)
Imidazolinones	*csr1-2*		*Arabidopsis thaliana*	Nuclear	Aragão et al. (2000))
Chlorsulfuron, Imazethapyr	*crs 1-4*		*Arabidopsis thaliana*	Nuclear	Ray et al. (2004)
Imidazolinone, sulfonylurea	*mals*		*Gossypium hirsutum*	nuvlear	Rajasekaran et al. (1996)
pyriminobac	*mals*		*Oryza sativa*	Nuclear	Wakasa et al. (2007)
Oxynils	*bxn*	Bromoxynil nitrilase	*Klebsiella pneumoniae subspecies ozanaenae*	Nuclear	Freyssinet et al. (1996)
Amiprophos-methyl, Chlorpropham, Pendimethalin, Norflurazon Acetochlor, Pendimethalin	*cyp1a1 cyp2c19 cyp2b6*	cytochrome P450 monooxygenase	*Homo sapiens*	Nuclear	Inui et al. (2005)
Butafenacil	*ppo*	protoporphyrinogen oxidase	*Arabidopsis thaliana*		Li et al. (2003)
			Myxococcus xanthus		Lee et al. (2007)
Bensulide	*opd*	organophosphate hydrolase	Pseudomonas diminuta	Nuclear	Pinkerton (2008)
Dinitroaniline	*TUAm*	mutant α-tubulin	Goosegrass (*Eleusine indica*)		Yemets et al. (2008)

[a]Table was adapted from Miki and McHugh (2004)

versions carrying one or more resistance mutations have been shown to be capable of conferring glyphosate tolerance in transformation selection. The utilization of a wild-type petunia EPSP synthase gene has been reported in transgenic petunia (Shah et al. 1986) and tobacco (van Bel et al. 2001) selection. A naturally glyphosate-resistant EPSP synthase gene from the *A. tumefaciens* strain CP4 has been used as a selective marker for transgenic soybean (Clementea et al. 2000), corn (Heck et al. 2005), wheat (Zhou et al. 1995; Hu et al. 2003), and tobacco (Ye et al. 2001). Mutant forms of the EPSP synthase genes (*aroA*) from *E. coli* (Della-Cioppa et al. 1987) or from *Salmonella typhimurium* (Comai et al. 1988) have been proved successful in selection of crop species such as cotton (Zhao et al. 2006), canola (Wang et al. 2006, 2008), and tobacco (Wang et al. 2003). Mutant maize (Howe et al. 2002) and rice (Charng et al. 2008) ESPSP genes also have been employed. The modified EPSPS enzymes encoded by these mutant genes have a decreased affinity for glyphosate while their kinetic efficiency is unaffected. In most of the cases mentioned earlier, the EPSP synthase gene was fused to a transit peptide sequence for chloroplast targeting. Working dosages of 20–200 μM glyphosate have been reported.

Glyphosate oxidase (GOX) is an enzyme that can break down glyphosate into two nontoxic compounds, aminomethylphosphonic acid (AMPA) and glyoxylate. A *GOX* gene cloned from *Ochrobactrum anthropi* strain LBAA (Barry et al. 1992) has been used as a selectable marker in tobacco, *Arabidopsis*, potato, and sugarbeet (Barry and Kishore 1995). A combination of CP4 EPSPS and GOX genes has been successfully used to transform wheat (Zhou et al. 1995) and sugarbeet (Mannerlöf et al. 1997).

Acetolactate Synthase

Acetolactate synthase (ALS, E.C. 4.1.8.13), also known as acetohydroxyacid synthase (AHAS), catalyzes the first reaction in the biosynthesis of the branched-chain amino acids isoleucine, valine, and leucine (Umbarger 1978). Inhibition of AHAS leads to the starvation of these amino acids in plants. The deficiency of these amino acids can also cause secondary effects such as accumulation of a toxic substrate (α-ketobutyrate), disruption of protein synthesis, and disruption of photosynthate transport. Eventually inhibition of AHAS leads to cell death and rapid growth cessation in susceptible species (Chaleff and Mauvais 1984; Ray 1984). Thechemical classes of commercial herbicides that can inhibit ALS include sulfonylureas (SU), imidazolinones (IM), triazolopyrimidines (TP), pyrimidinyl thiobenzoates (Saari et al. 1994 and references therein), and sulfonylamino-carbonyl-triazolinones (Santel et al. 1999). Plant species differ in herbicide susceptibility and can develop resistance to different classes of AHAS inhibitors. In most cases, resistance to AHAS-inhibiting herbicides, in otherwise susceptible species, is caused by point mutations in AHAS genes that reduce the affinity of the enzyme to herbicide inhibition (Kolkman et al. 2004). Consequently, the enzymatic pathway will continue to work, making the plants resistant to the herbicide. The mutant forms of plant AHAS can act as effective

selectable marker genes when combined with AHAS-inhibiting herbicide. Mutated-AHAS (mAHAS) genes have been isolated from a number of resistant plant genomes, such as *Arabidopsis* (Haughn and Somerville 1986), lettuce (Eberlein et al. 1999), and rice (Wakasa et al. 2007). The most commonly used mAHAS genes are *crs1* genes isolated from mutants of *Arabidopsis thaliana*, which are resistant to sulfonylurea and imidazolinone herbicides. These *csr*1 genes have been used in selection of several transgenic plant species, such as rice (Li et al. 1992), tobacco (Charest et al. 1990), maize (Fromm et al. 1990), canola (Miki et al. 1990), common bean (Bonfim et al. 2007), soybean (Aragão et al. 2000), potato (Andersson et al. 2003), poplar (Brasileiro et al. 1992), and jujube (*Zizyphus jujuba* Mill.) (Gu et al. 2008). It was found that an *Arabidopsis* double mutant (two mutation points) gene (*crs 1-4*) seems to provide more efficient selection than most single mutant genes (Ray et al. 2004). The mAHAS genes from rice (Wakasa et al. 2007) and cotton (Rajasekaran et al. 1996) have also been successfully used as selective marker.

Bromoxynil-Specific Nitrilase

Oxynil herbicides are phenolic molecules that inhibit photosynthesis in plants by binding to electron-transport components of photosystem II in the thylakoid membrane. Two oxynil herbicides are available: bromoxynil and ioxynil. The bromoxynil nitrilase (*bnx)* gene from *Klebsiella ozaenae* codes for a bromoxynil-specific nitrilase 3,5-dibromo-4-hydroxybenzonitrile aminohydrolase (E.C. 3.5.5.6) that hydrolyzes bromoxynil into 3,5-dibromo-4-dihydroxybenzoic acid and ammonia (Stalker et al. 1988), thus confers resistance to bromoxynil. Successful transformation using the *bnx* gene as a selectable marker has been reported in tobacco and canola without using other selectable markers (Freyssinet et al. 1996; Warwick and Miki 2004). However, the *bnx* gene has not been widely used. Cereal plants and several other monocotyledonous crops such as onions are naturally resistant to oxynil herbicides because they are able to metabolize the molecule to the non-phytotoxic benzoic acid (Freyssinet et al. 1996). Thus *bxn* is not a suitable selectable marker for the transformation of monocotyledonous species.

Cytochrome P450 Monooxygenase

P450 monooxygenases are heme proteins that use electrons from NADPH to catalyze the activation of molecular oxygen. Mammalian P450 species show overlapping and broad substrate specificity and confer the ability to metabolize a number of chemicals, including herbicides. Most classes of herbicides are aryl- or alkyl-hydroxylated or *N*-, *S*-, or *O*-dealkylated by P450 species. The phenylurea herbicide chlortoluron is detoxified either via hydroxylation of the ring-methyl or via di-*N*-demethylation (Gonneau et al. 1988). Human P450 species have been used to generate herbicide-tolerant tobacco, potato, and rice plants (Shiota et al. 1994; Inui et al. 2000, 2001). The feasibility of human P450 as a selectable marker has

been tested in *Arabidopsis*. A combination of chlorpropham and amiprophos-methyl resulted in a transformation rate that was equal to that of kanamycin selection in the transgenic plants expressing *CYP1A1* and *CYP2C19* cDNAs, respectively (Inui et al. 2005).

Protoporphyrinogen Oxidase

Protoporphyrinogen oxidase (PPO, EC 1.3.3.4), a key enzyme in the chlorophyll/heme biosynthetic pathway, catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX (Smith et al. 1993). Inhibition of PPO by the PPO family of herbicides, e.g., butafenacil, causes accumulation of protoporphyrin IX, which then causes light-dependent membrane damage (Lee et al. 1993). Plants overexpressing native PPO genes or naturally tolerant PPO showed resistance to diphenyl ether herbicide (Li et al. 2003 and references therein). A *PPO* double mutant gene was cloned from *Arabidopsis* (Li et al. 2003). In combination with butafenacil, the *Arabidopsis* mutated *PPO* proved to be an effective selectable marker in maize transformation, with transformation frequency comparable to *pat* and *pmi* systems (Li et al. 2003). A *Myxococcus xanthus* native *PPO* (*Mx PPO*, under the control of the constitutive maize ubiquitin promoter) and butafenacil (0.1 μM) selection system was recently demonstrated in rice (Lee et al. 2007).

Organophosphate Hydrolase

Bensulide herbicide is a lipid synthesis inhibitor (not at the acetyl CoA carboxylase site) (Prather et al. 2002). This herbicide can cause precocious vacuolization of meristem cells and inhibit shoot and root development (Cutter et al. 1968). A bacterial organophosphate hydrolase (OPH; EC 3.1.8.1) gene has been recently tested in maize. The encoded enzyme hydrolyzes the toxic organophosphate. It was suggested that this OPH gene may serve as screenable as well as scorable maker (Pinkerton 2008).

Mutant α-Tubulin Genes

Tubulin is the main protein component of microtubules. Antimicrotubule herbicides, such as dinitroanilines and phosphoroamidates, can directly poison microtubule dynamics in plant cells, which results in the cessation of mitosis (Morejohn and Fosket 1984). Thus, antimicrotubule herbicides have been used for chromosome doubling (see for review Khosravi et al. 2007). At higher concentration levels, this type of herbicide inhibits callus growth and plant regeneration and can serve as strong selection agents (see for review Sundar and Sakthivel 2008). The feasibility of using antimicrotubule herbicides as selective reagents in plant transformation has been explored both in monocots and dicots (see for review in Sundar and Sakthivel

2008). According to Yemets et al. (2008), a selection scheme with a combination of a mutant α-tubulin gene from goosegrass (*Eleusine indica*) and dinitroaniline generated an efficiency of transgenic plant selection that was comparable with those using kanamycin or PPT. The effective concentrations of trifluralin range from 3 to10 μM, depending on the species.

4.2.2.3 Other Toxic Compounds (Table 4.7)

2-Deoxyglucose

2-Deoxyglucose (2-DOG) is an analog of glucose. In the cytosol of plant cells, 2-DOG is phosphorylated by hexokinase yielding 2-DOG-6-phosphate (2-DOG-6-P). 2-DOG-6-P acts as a competitor of glucose-6-phosphate. 2-DOG-6-P is known to severely impair plant growth due to multiple effects in metabolism. In addition to inhibiting glycolysis and overall protein synthesis, it interferes with the glycosylation of proteins and the synthesis of cell wall polysaccharides (Kunze et al. 2001 and references therein). Two yeast genes encoding 2-deoxyglucose-6-phosphate phosphatase (EC 3.1.3.68) (DOG^R1 and DOG^R2) have been identified. When overexpressed in yeast, DOG^R1 and DOG^R2 conferred 2-DOG resistance (Randez-Gil et al. 1995). Selection based on yeast DOG^R1 and 2-DOG has been demonstrated successfully in pea (Sonnewald and Ebneth 2004), potato, and tobacco (Kunze et al. 2001). Whereas the use of this selection scheme resulted in lower efficiency for transgenic tobacco plants than the *nptII* gene, comparable efficiency was achieved in the selection of transgenic potato (Kunze et al. 2001). It was also reported that 2-deoxyglucose-6-phosphate phosphatase has narrow substrate specificity and no abnormalities were observed in the transgenic plants.

Betaine Aldehyde Dehydrogenase (BADH)

Betaine aldehyde is phytotoxic to many plant cells and has an adverse effect on growth. According to a study conducted by Rathinasabapathi et al. (1994), shoot regeneration from tobacco leaves, cotyledon expansion, and greening in germinating seedlings were severely inhibited in the presence of 5 mM betaine aldehyde. Genes encoding betaine aldehyde dehydrogenase (BADH, EC 1.2.1.8) have been cloned from several plant species, including spinach, sugarbeet, and amaranth. BADH is highly specific for betaine aldehyde and converts it to glycine betaine, which accumulates in a few crop species as an osmoprotectant. *BADH* is well suited as a chloroplast selectable marker gene. Expression in the chloroplast allowed direct selection and regeneration of transgenic tobacco plants in the presence of betaine aldehyde with an efficiency that was 25-fold higher than spectinomycin resistance conferred by the *aadA* gene (Daniell et al. 2001). But when expressed in the nuclear genome of tomato, *BADH* was not as effective as

Table 4.7 Negative selection based on other toxic compounds[a]

Drugs and analogs	Genes	Enzymes	Sources	Genome	References
2-Deoxyglucose	*dogr1*	2-Deoxyglucose-6-phosphate phosphatase	*Saccharomyces cerevisiae*	Nuclear	Kunze et al. (2001)
Betaine aldehyde	*badh*	Betaine aldehyde dehydrogenase	*Spinacia oleracea*	Nuclear plastid	Ursin (1996)
S-Aminoethyl l-cysteine (AEC)	*dhps*	Dihydrodipicolinate synthase	*Escherichia coli*	Nuclear	Perl et al. (1993)
Lysine and threonine	*ak*	Aspartate kinase	*Escherichia coli*	Nuclear	Perl et al. (1993), Tewari-Singh et al. (2004)
4-Methyltryptophan (4-mT)	*tdc*	Tryptophan decarboxylase	*Catharanthus roseus*	Nuclear	Goddijn et al. (1993)
Methotrexate	*dhfr*	Dihydrofolate reductase	*Escherichia coli, mouse*	Nuclear	Herrera-Estrella et al. (1983), Eichholtz et al. (1987)
			Candida albicans	Nuclear	Irdani et al. (1998)
5-Methyl-tryptophan (5-mT), CdCl2	*tsb1*	Tryptophan synthase beta 1	*Arabidopsis thaliana*	Nuclear	Hsiao et al. (2007)
Actinonin	*def2-d*	Peptide deformylase	*Arabidopsis thaliana*	Nuclear	Hou et al. (2007)
L-O-Methylthreonine (OMT)	*ilva omr1-5, omr1-7, and omr1-8*	Threonine deaminase gene	*Escherichia coli, Arabidopsis thaliana*	Nuclear	Ebmeier et al. (2004), Garcia and Mourad (2004))
4-Methylindole (4MI), 5-Methyltrypthopan (5MT), and 7-Methyl-DL-tryptophan (7MT)	*asa1d* *asa2*	Anthranilate synthase	*Oryza sativa* *Nicotiana tabacum*	Nuclear	Yamada et al. (2005) Barone and Widholm (2008)
D-Serine	*dsda*	D-serine ammonia lyase	*Escherichia coli*	Nuclear	Erikson et al. (2005)
D-Serine, D-alanine, D-isoleucine, D-valine	*dao1*	D-amino acid oxidase	*Rhodotorula gracilis*	Nuclear	Erikson et al. (2004)

Gabaculine	*heml*	Glutamate-1-semialdehyde aminotransferase	*Synechococcus PCC6301*	Nuclear	Gough et al. (2001)
Galactose	*galT*	UDP-glucose:galactose-1-phosphate uridyltransferase	*Escherichia coli*	Nuclear	Joersbo et al. (2003)
Cyanamide	*cah*	Cyanamide hydratase	*Myrothecium verrucaria*	Nuclear	Damm (2003), Weeks et al. (2000)
S-aminoethyl l-cysteine (AEC), homo-arginine	*ocs*	Octopine synthase	*Agrobacterium tumefaciens*	Nuclear	Koziel et al. (1984)

[a]Table was adopted from Miki and McHugh (2004)

*nptII (*Ursin 1996). *BADH* also has been used for salt resistance in several crops, e.g., tomato (Jia et al. 2001) and tobacco (Rathinasabapathi et al. 1994; Liang et al. 1997).

Dihydrodipicolinate Synthase and Aspartate Kinase

Dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) and aspartate kinase (AK, EC 2.7.2.4) are key enzymes in the aspartate family pathway, which leads to the biosynthesis of lysine, threonine, methionine, and isoleucine. Both enzymes are feedback-regulated: aspartate kinase is feedback-inhibited by lysine and threonine (LT), while dihydrodipicolinate synthase is inhibited by lysine or its toxic analog S-aminoethyl l-cysteine (AEC). Growth in the presence of millimolar concentration of LT causes methionine starvation due to the complete inhibition of aspartate kinase activity by these two amino acids and results in strong inhibition of growth in a wide range of plant species (Rognes et al. 1983; Arruda et al. 1984; Miao et al. 1988). The DHDPS enzymes from *E. coli* are less sensitive to feedback inhibition. Tobacco plants expressing a bacterial dihydrodipicolinate synthase gene in their chloroplasts had an increased production of LT (Shaul and Galili 1991). When controlled by the CaMV 35S promoter, a bacterial desensitized aspartate kinase gene has been successful as a selectable marker for use in the production of transgenic potato (Perl et al. 1993) and chickpea (Tewari-Singh et al. 2004), coupled with 2 mM of each of LT. A selection system with a bacterial dihydrodipicolinate synthase gene and 0.15 mM AEC was also successful in production of transgenic potato (Perl et al. 1993). One of the potential drawbacks is that the overproduction of lysine or threonine resulting from the modification of metabolism causes abnormalities in some plants (Perl et al. 1993).

Tryptophan Decarboxylase

The enzyme tryptophan decarboxylase (TDC; EC 4.1.1.28) catalyzes the conversion of L-tryptophan into tryptamine (Noé et al. 1984), which is an intermediate in the biosynthesis of terpenoid indole alkaloids. Besides L-tryptophan, toxic compounds like 4-methyltryptophan (4-mT), 4-fluorotryptophan, and 5-fluorotryptophan can be substrates of TDC. When a *Catharanthus roseus* gene coding for TDC was placed under the control of the CaMV 35S promoter and introduced into tobacco, direct selection on 0.1-mM 4-mT yielded transgenic plants with the same efficiency as the *nptII* gene (Goddijn et al. 1993), and the transgenics appeared normal in the greenhouse. To date, this selection system has not been widely employed. A possible disadvantage associated with this *TDC*/4-mT selection scheme is the accumulation of tryptamine in the transformed tissue. The applicability of this selection system in other plant species will depend on their

endogenous tryptophan decarboxylase activity as well as their tolerance to elevated TDC-directed tryptamine levels.

Dihydrofolate Reductase

Dihydrofolate reductase (DHFR, E.C. 1.5.1.3) plays an essential role in the metabolic pathways of adenine, histidine, methionine, and thimidilate biosynthesis by reducing dihydrofolate to tetrahydrofolate. Antifolate drugs, such as trimethoprim and methotrexate (Mtx), can bind very tightly to the active site of the enzyme DHFR, therefore impairing protein, RNA, and DNA biosynthesis and, consequently, leading to cell death (Habert et al. 1981). Production of transgenic plants by using a mouse or a fungal dihydrofolate reductase gene (*dhfr*) as a new selectable marker and methotrexate (Mtx) as selection agent was successful in tobacco (Irdani et al. 1998; Aionesei et al. 2006), canola (Pua et al. 1987), and petunia (Eichholtz et al. 1987). A high transformation rate of 10% was reported in canola (Pua et al. 1987). Plant cells are generally very sensitive to low levels of Mtx presumably because of the inherent low activity of the enzyme (Ratnam et al. 1987). Methotrexate dosage ranges of 3–100 ng mL^{-1} have been used.

Tryptophan Synthase β1

5-methyl-tryptophan, an analog of the essential amino acid tryptophan, is toxic to plants, since it inhibits tryptophan biosynthesis. Hsiao et al. (2007) recently reported that enhanced expression of *Arabidopsis* tryptophan synthase (EC 4.2.1.20) **β**1 (*AtTSB1*) and the use of 5-methyl-tryptophan and/or $CdCl_2$ as selection agent(s) yielded comparable transformation efficiency in *Arabidopsis* to the conventional hygromycin selection system (Hsiao et al. 2007). Thus, the TSB1 system provides a novel selection system. In addition, overexpression of *AtTSB1* in *Arabidopsis* and tomato confers tolerance to cadmium stress (Hsiao et al. 2008).

Peptide Deformylase

Peptide deformylase (DEF, EC 3.5.1.88) catalyzes the removal of the *N*-formyl group from the initiating methionine in newly translated proteins, thus is essential for all subsequent *N*-terminal protein processing as well as cell survivability. Actinonin, a specific inhibitor of peptide deformylase, has broad-spectrum herbicidal activity against a wide range of plants, including many agriculturally important weed species (Hou et al. 2007). Actinonin has been reported to cause chlorosis and severe inhibition of growth and development, thus having profound herbicidal effects when applied to many plant species both pre- and postemergence (Hou et al. 2006). A direct selection system with an *Arabidopsis Atdef2-D* and

actinonin (1.2 mM actinonin) yielded transformation efficiency equal to kanamycin in tobacco (Hou et al. 2007). Cotyledons of plants expressing the *AtDEF2* transgene remained white, but all subsequent growth was normal.

Threonine Deaminase

Threonine deaminase (TD, EC 4.2.1.16) catalyzes the initial step in the synthesis of isoleucine (Ile) by deaminating of threonine to 2-ketobutyrate and ammonia. This enzyme is feedback-regulated by Ile. Overexpression of wild-type or Ile-insensitive mutant threonine deaminase genes in planta increases cellular concentrations of Ile (Slater et al. 1999) and provides resistance to L-O-methylthreonine (OMT) (Mourad et al. 1995; Garcia and Mourad 2004). A structural analog of Ile, OMT is able to compete effectively with Ile during translation and induce cell death. When coupled with OMT as the selection agent, an *E. coli* wild-type threonine deaminase gene, *ilvA,* could be utilized as a selectable marker to identify tobacco transformants (Ebmeier et al. 2004). However, the transformation efficiency was substantially lower than that observed with *nptII* using kanamycin as the selection agent. In addition, a severe off-phenotype was observed under greenhouse conditions, which correlated with increased levels of expression of the *ilvA* transgene in a subset of the transformants. The *Arabidopsis* mutant, feedback-insensitive threonine deaminase alleles (*omr1-5*, *omr1-7*, and *omr1-8*) may serve as better selectable markers since *Arabidopsis* plants transformed with either of these mutant genes had a normal phenotype, undistinguishable from wild-type (Garcia and Mourad 2004). However, direct selection with these *Arabidopsis* genes and OMT needs to be further demonstrated in crop species.

Anthranilate Synthase

Anthranilate synthase (AS, EC 4.1.3.27) is the first enzyme in the tryptophan biosynthetic pathway and catalyzes the conversion of chorismate to anthranilate. Its catalytic activity is regulated by feedback inhibition of tryptophan (Trp). Feedback-insensitive forms of AS have been found in a number of cell lines of crop species, including tobacco, rice, and potato (Barone and Widholm 2008 and references therein). These cell lines showed resistance to toxic Trp analogs, such as 4-methylindole (4MI), 5-methyltrypthopan (5MT), and 7-methyl-DL-tryptophan (7MT). The feasibility of the selection system with the feedback-insensitive anthranilate synthase α-subunit (*ASA*) gene in combination with the use of Trp or indole analogs as selective agent has been demonstrated in potato, rice, and tobacco (Yamada et al. 2005; Barone and Widholm 2008). Transformed plants grew normally, and a dosage of 300 μM Trp analog was proved effective for selection. Selection with AS system was as effective as hygromycin B selection in rice (monocotyledon) and kanamycin selection in potato (dicotyledon), according to Yamada et al. 2005.

D-Amino Acid Deaminase

Plants have low capacity for D-amino acid metabolism and several D-amino acids are toxic to plants even at relatively low concentrations (Brückner and Westhauser 2003; Forsum et al. 2008). Using D-amino acids and the *dsdA* (D-serine ammonia lyase) gene from *E. coli* and the *dao1* (D-amino acid oxidase) from yeast *Rhodotorula gracilis* as selectable makers has been evaluated in recent years. The *dsdA* gene encodes D-serine ammonia lyase (EC 4.3.1.19), which catalyzes the deamination of D-serine into pyruvate, water, and ammonium. D-amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the oxidative deamination of a range of D-amino acids (Alonso et al. 1998), including the toxic D-serine and D-alanine. When driven by the CaMV 35S promoter, the bacterial *dsd1* and the yeast *dao1* provided efficient selection in *Arabidopsis* and maize transformation (Erikson et al. 2004, 2005; Lai et al. 2006), coupled with appropriate D-amino acids. Transgenic plants did not exhibit any adverse phenotypes. Both *dsdA* and *dao1* markers allowed flexibility in application of the selective agent in *Arabidopsis*: it can be applied in sterile plates, in foliar sprays, or in liquid culture (Erikson et al. 2004, 2005). The yeast DAAO was able to metabolize nontoxic D-amino acids D-isoleucine and D-valine into toxic compounds and killed transgenic *Arabidopsis* (Erikson et al. 2004). Considering that the natural occurrence of D-amino acids in plants is generally low, especially with no detectable levels of D-valine and D-isoleucine (Brückner and Westhauser 2003; Forsum et al. 2008), *dao1* gene can serve as a substrate-dependent, dual-function, selectable marker in plants.

Glutamate-1-semialdehyde Aminotransferase

Glutamate-1-semialdehyde aminotransferase (GSA-AT, EC. 5.4.3.8) is involved in the C5 pathway and catalyzes the conversion of glutamate-1-semialdehyde into aminolaevulinic acid (ALA). Gabaculine (3-amino-2,3-dihydrobenzoic acid) is a bacterial phototoxin, an irreversible inhibitor of a wide range of pyridoxal-5-phosphate-linked aminotransferases (Rando 1977). A mutant form of GSA-AT, encoded by the *hemL* gene, was discovered in a gabaculine-resistant cyanobacterium, *Synechococcus* PCC6301 strain GR6 (Grimm et al. 1991) and utilized in transformation of tobacco and alfalfa as a selectable marker, where it was driven by the double CaMV 35S promoter and targeted to chloroplasts with the transit peptide of the ribulose bisphosphate carboxylase small subunit (Gough et al. 2001; Rosellini et al. 2007). Gabaculine could be applied in media or by spray. According to Rosellini et al. (2007), the gabaculine-based system is more efficient than the conventional, kanamycin-based system. The inheritance of *hemL* was Mendelian, and no obvious phenotypic effect of its expression was observed.

Galactose-1-phosphate Uridyltransferase

Galactose has long been known to be toxic to a broad range of plant species (Joersbo et al. 2003). The toxicity of galactose is believed to be due to accumulation of galactose-1-phosphate, generated by endogenous galactokinase after uptake. An *E. coli* UDP-glucose:galactose-1-phosphate uridyltransferase (EC 2.7.7.12) (*galT*) gene, driven by a CaMV 35S promoter, was found to allow transgenic shoots of potato and canola to regenerate on galactose-containing selection media, resulting in high transformation frequencies (up to 35% for potato with 1.25 g L^{-1} galactose) (Joersbo et al. 2003). However, use of the *galT*/galactose selection system did not promote regeneration of transgenic apple plants (Degenhardt et al. 2007).

Cyanamide Hydratase

Cyanamide is a nitrogen-rich compound that has been used as a nitrogen fertilizer, defoliant, and herbicide. Due to its toxicity, cyanamide has also been used as a selection agent for plant transformation paired with a gene for the enzyme cyanamide hydratase (E.C. 4.2.1.69) (*Cah*) isolated from the soil fungus *Myrothecium verrucaria* (Maier-Greiner et al. 1991). This enzyme converts cyanamide to the common metabolite urea and has an extremely narrow substrate specificity. The *Cah* gene and cyanamide selection has been used to select transformants of wheat, tobacco, potato, rice, sorghum, soybean, and tomato (Miki and McHugh 2004; Ulanov and Widholm 2007).

Octopine Synthase

Octopine synthase (also called lysopine dehydrogenase) catalyzes the synthesis of opines by the reductive condensation of certain amino acids with pyruvate. This enzyme also metabolizes lysine toxic analog S-aminoethyl l-cysteine (AEC) and toxic arginine analog homo-arginine. The gene encoding octopine synthase is part of the T-DNA component of the *A. tumefaciens* octopine Ti plasmids. Dahl and Tempe (1983) found that callus tissues expressing the enzyme appear to be 20-fold more tolerant to AEC. Selective growth of callus on AEC or homo-arginine was shown in experiments with petunia stem explants (Koziel et al. 1984) or tobacco (Van Slogteren et al. 1982). This selection scheme has not been widely adopted.

4.3 Screening Strategies

Transgenic plants are generally developed by coinsertion of a selectable marker gene with a gene of interest. Putative transgenic plants selected for antibiotic resistance further need to be evaluated for integration of the transgene and its

expression because some cells may escape from the effect of antibiotics during tissue culture. Diffusion of selectable marker gene product to neighboring cells may facilitate nontransgenic cells to survive the selection pressure and regenerate. In addition, expression of the gene of interest also varies among different integration events. This necessitates a screening strategy that can confirm integration and expression of the gene of interest. Use of reporter genes (*cat, lacZ, uidA, luc, gfp*) allows discrimination of transformed and nontransformed plants and also monitoring their expression. By employing vital marker (*luc*, *gfp*) gene expression, protein localization and intracellular protein traffic can be observed in situ, without destroying the plants (Ziemienowicz 2001). Commonly used reporter genes have been summarized in Table 4.8.

4.3.1 Scorable Markers

A fundamental difference between a selectable marker gene and a reporter gene is that selectable markers allow transgenic cells to survive and multiply at lethal concentrations of a selective agent (e.g., antibiotic), while scorable markers produces distinct phenotype that can be easily identified in the background of nontransformed cells (e.g., GUS, GFP, luciferase) (Miki and McHugh 2004). Reporter genes encode proteins that can be detected directly (e.g., GFP), or they catalyze specific reactions the products of which are detectable (e.g., GUS and luciferase).

An ideal scorable marker should have certain desirable features such as, (1) Availability of sensitive detection system with a high signal-to-noise (endogenous background) ratio; (2) Reporter signal should be measurable quantitatively; (3) Reporter gene products should be resistant to chemicals and processes used in histological processing; (4) Histochemical assays should have low diffusion of assay products across the neighboring cells; (5) Reporter gene products should have a short half-life so that it gives a true representation of transcription activity of a cell; and (6) Assays should be nondestructive to plant tissue. Although none of the currently used reporter systems have all these features, a suitable reporter gene can be selected on the basis of experimental requirements.

4.3.1.1 β-Galactosidase

LacZ gene of *E. coli* that encodes β-galactosidase is the most extensively used reporter gene system in microorganisms and animals. β-galactosidase (E.C. 3.2.1.23) a tetramer in its active form has a molecular weight of 116 kDa and optimum pH 7.0–7.5 for its activity. *LacZ* reporter gene has also been used in plant systems (Helmer et al. 1984), but its applications have been hampered by the presence of endogenous galactosidase (David et al. 1998; Stano et al. 2002; Esteban et al. 2005), which is active at pH 7–7.5 suitable for LacZ activity. Consequently

Table 4.8 Genes for the screening of transgenic tissues/plants

Substrates	Genes	Enzymes/protein	Sources	Genome	References
X-Gal	*LacZ*	β-galactosidase	*E. coli*	nuclear	Helmer et al. (1984)
X-Gluc, 4-MUGIuc	*GUS* (*uidA*)	β-glucuronidase	*E. coli*	nuclear	Jefferson et al. (1987)
Luciferin/ coelenterazine	*LUC*/ ruc	Luciferase	*Photinus pyralis*/ *Renilla reniformis*	nuclear	Ow et al. (1986)
None	*GFP*	Green fluorescent protein	*Aequorea victoria*	nuclear	Chalfie et al. (1994)
oxalic acid+ N,N-dimethylaniline + 4-aminoantipyrine	*OxO*	Oxalate oxidase	*Triticum aestivum*	nuclear	Simmonds et al. (2004)
None	*R, C1* and *B*	R, C1 and B transcription factors	*Zea mays*	nuclear	Ludwig et al. (1990)
None	*Phytoene synthase*	Phytoene synthase	*Lycopersicon esculentum*	nuclear	Trulson et al. (1997)
None	*AsRed, AmCyan, ZsYellow, ZsGreen DsRed eqFP611*	Reef Coral Fluorescent Proteins	*Anemonia sulcata* *Anemonia majano* *Zoanthus* sp. *Zoanthus* sp. *Discosoma* sp. *Entacmaea quadricolour*	nuclear	Wenck et al. (2003)
Kanamycin + (^{32}P) ATP	*NPTII*	neomycin phosphotransferase II	*Escherichia coli* K12	nuclear	Fregien and Davidson (1985)
Ariginine + pyruvate + NADH	*NOS, OCS*	nopaline synthase, octopine synthase	*Agrobacterium tumefaciens*	nuclear	Johnson et al. (1974)
(^{14}C) chloramphenicol + acetyl-coA	*CAT*	Chloramphenicol acetyltransferase	*E. coli* Tn9	nuclear	Herrera-Estrella et al. (1984)

the *LacZ* reporter gene has not been as widely used in plants as in microorganisms and animals.

β-galactosidase can be assayed directly in plant extracts when they contain high levels of LacZ expression. At low levels of expression, endogenous galactosidase and LacZ activities are separated electrophoretically followed by detection of the enzymes with a fluorogenic substrate (4-methyl umbelliferyl-3-D-galactoside). LacZ protein is resistant to various physiochemical factors. Tissue containing LacZ can be fixed with glutaraldehyde without loss of activity. These factors make *LacZ* a good reporter gene for histochemical analysis (Teeri et al. 1989).

4.3.1.2 GUS, β-Glucuronidase (*uidA*)

Among all reporter genes developed so far, *E. coli* gene *uidA*, encoding β-glucuronidase (GUS, E.C. 3.2.1.31) has been the most extensively used reporter gene in transgenic plants. The β-glucuronidase is a homotetramer with a molecular mass of approximately 68 kDa and a pH optimum of 7–8. The *uidA* reporter system possesses most of the features required for use in plants, such as ease of assay, high sensitivity, sufficient specificity of the enzymatic reaction, possibility of histochemical localization, activity of the enzyme in translational fusions, and availability of substrates for spectrometric, fluorometric, and histochemical assays (Jefferson et al. 1987). There is little or no detectable β-glucuronidase activity in almost any higher plant at pH levels used in the assay and endogenous GUS activity is abolished by including methanol in the assay buffer (Hu et al. 1990; Kosugi et al. 1990) (Fig. 4.1a).

For spectrometric, fluorometric, and histochemical assays, p-nitrophenyl-p-D-glucuronide, 4-methylumbelliferyl glucuronide (4-MUGIuc), and 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) are commonly used substrates. β-glucuronidase cleaves 4-methylumbelliferyl glucuronide (4-MUGIuc) to fluorescent compound 4-methylumbelliferon, while the colorless, water-soluble product of the enzymatic cleavage of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) undergoes an oxidative dimerization to yield an indigo blue precipitate. The disadvantage of all the GUS assays is that the plant tissue has to be discarded.

One way of performing a nondestructive GUS assay is to test the excreted β-glucuronidase in liquid plant culture media or wound exudates, with florescent substrate 4-MUGIuc. In another viable test, either X-Gluc is applied to solid growth media for root staining or 4-MUGIuc is sprayed on β-glucuronidase expressing leaves (Martin et al. 1992). This GUS assay can be used for screening large transgenic populations, but high toxicity of X-Gluc and weak detection limit its applications.

While the stability of GUS protein is a major advantage to analyze the activities of weak promoters, it can interfere with the correct interpretation of promoter reporter expression data. A rapid change in the transcription of the reporter gene may not be reflected in corresponding rapid changes in GUS activity (Taylor 1997). Secondly, 5-bromo-4-chloro- 3-indoxyl (X-gluc product) diffuses into the

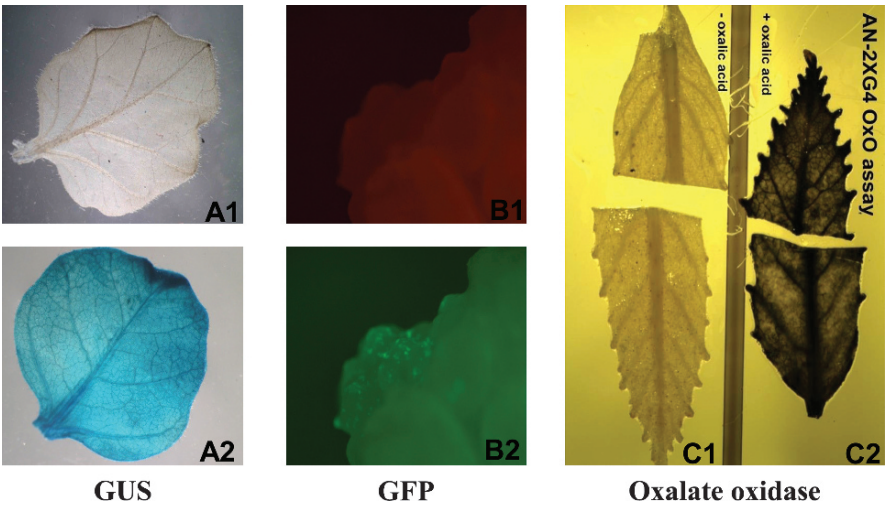

Fig. 4.1 (**a**) Histochemical analysis of GUS activity driven by the CaMV 35S promoter in nontransgenic (A1) and transgenic eggplant (*Solanum melongena*) (A2) (V Nain, unpublished). (**b**) GFP transformed cells (green spots) on American chestnut (*Castanea dentata*) somatic embryos under different lights and filters (WA Powell & CA Maynard, unpublished). Chlorophyll emits red florescence at 680 nm (B1) while green florescence is emitted at 522 nm (B2) that can be distinguished from background. (**c**) An oxalate oxidase assay: without oxalic acid (negative control) (C1) and with oxalic acid added in assay buffer (C2). This is of tissue culture leaves from transgenic American chestnut transformed with OxO transgene driven by a vascular promoter (LCG Northern, CA Maynard, and WA Powell, unpublished)

neighboring cells, where it precipitates giving a false GUS expression signal in the nonexpressing cells (Mascarenhas and Hamilton 1992). Several reports involving analysis of temporal expression patterns (Hird et al. 1993; Caissard et al. 1994; Treacy et al. 1997; Ariizumi et al. 2002; de Ruijter et al. 2003) have commented on the possible errors in deducing activities using GUS as a reporter. Although technical solutions have been proposed to alleviate these artifacts (De Block and Debrouwer 1993), they have not always been found to be adequate (de Ruijter et al. 2003; Kavita and Burma 2008).

Another artifact in GUS assay arises with residual *Agrobacterium* cells (harboring GUS construct) that have been used for infection of explants, in a genetic transformation experiment. Leaky GUS expression in *Agrobacterium* cells stains blue in a GUS assay, which in turn gives a false GUS signal to the plant tissue as well. This problem is circumvented by inserting an intron (with a stop codon) in the coding region of GUS. As *Agrobacterium* does not have a splicing mechanism and the intron has a stop codon in the reading frame, GUS transcripts formed by leaky expression in *Agrobacterium* do not translate into functional GUS enzyme. Whatever GUS activity is detected comes from the plant tissue only. The pCAMBIA series of vectors such as pCAMBIA1201; 1301; 2201; and 2301 consist of an intron (from the castor bean catalase gene) in the coding sequence to ensure that the activity is derived only from β- Glucuronidase expressed in the plant cell.

4.3.1.3 Luciferase

The search for a reporter gene that could be used in viable plant tissues and whose signal is representative of changes in transcriptional activity of the gene at any given time, culminated with the development of the North American fire fly (*Photinus pyralis*) luciferase (LUC) gene as a reporter system (Ow et al. 1986). The enzyme luciferase (E.C. 1.13.12.7) confers on the organism the ability to glow (exhibits luminescence at 562 nm) in the dark. In the first step, luciferase catalyzes the oxidative carboxylation of luciferin (6-hydroxy-benzothiazole) to excited form of oxyluciferin in the presence of ATP, Mg^{2+}, and O^2. The reaction produces a light flash at a maximum of 562 nm.

Hydrozoan's (*Renilla reniformis*) luciferase is encoded by the *ruc* gene. When used with luciferin and ATP, firefly luciferase/luciferin emits light at 560 nm, while *Renilla* luciferase/coelenterazine emits light at 475 nm. Because these two reporter systems emit light at quite different wave lengths, it is possible to use firefly luciferase/luciferin and *Renilla* luciferase/coelenterazine as a dual reporter system. Luciferases have been isolated from bacteria (*Vibrio harveyi*) also. The bacterial luciferase (LUX, E.C. 1.14.14.3) is a heterodimer, with two peptide subunits encoded by genes *lux* A and *lux* E, while firefly luciferase consists of a single polypeptide encoded by a gene *luc*.

There are two different methods of luciferase substrate application to plant tissues. In in vivo methods plants are grown in substrate supplemented medium, so that the substrate is absorbed through roots and gets distributed in the plant. This method needs extensive physical handling and a long time period for plant growth. It also limits the size of plant that can be analyzed. In the second method luciferin solution containing a mild detergent is sprayed on leaves. It requires only 10-min incubation time before measuring the light emission.

Advantages of the *luc* reporter gene are that it is not destructive to the plant tissue and has a short half-life in vivo that generally reflects real-time gene expression status in the transgenic tissue under investigation. Because of the high reaction efficiency of the firefly luciferase, this reporter gene is excellent for screening purposes.

4.3.1.4 GFPs

Since the first reports of *Aequorea victoria* green fluorescent protein (AvGFP) as a reporter gene in the nematode *Caenorhabditus elegans* (Chalfie et al. 1994), it has found wide applications in transgenic plants. O. Shimomura, M. Chalfie, and R. Y. Tsien won the 2008 Nobel prize in chemistry for discovery and development of GFP. Its high sensitivity, absence of external substrate application, and viability of the tissue under testing make it an ideal reporter gene (Fig. 4.1b). GFPs have several advantages over the previously utilized markers for transformation, gene expression, and protein localization studies (Stewart 2001, 2006).

Because of the presence of a cryptic splice site, native AvGFP does not express up to its detection limit in plants (Haseloff et al. 1997). Removal of the splice site and

targeting to endoplasmic reticulum resulted in high expression of AvGFP in plants. Another green fluorescent protein, AcGFP, has been isolated from a nonbioluminescent jellyfish (*Aequorea coerulescens*) (Gurskaya et al. 2006). Moreover, a full range of color variants of fluorescent proteins are now available for transformation, with the opportunity to further customize fluorescence to specific applications (see Sect. 3.1.5 and reviews by Galbraith 2004 and Stewart 2006). Synthetic GFP (*sgfp*) genes that have codon usage and RNA stability optimized for plant expression are even better reporters, especially in comparison to GUS, for assessment of the temporal activities of promoters that have very narrow windows of expression. This is due to the short half-life of the GFP protein (18 h) (de Ruijter et al. 2003) as compared with that of GUS, that has a half-life of 3–4 days in tobacco plants (Weinmann et al. 1994) and about 50 h in tobacco protoplasts (Jefferson 1987; Jefferson et al. 1987).

Another application of GFP is in the determination of zygosity of transgenic plants. Fluorescent proteins can provide instantaneous data on homo- or heterozygosity. Halfhill et al. (2003) have found that heterozygous (hemizygous) transgenic canola plants exhibit half of the green fluorescence of homozygous plants and expression levels are inherited quantitatively at the same heterologous level of florescence in wild relatives also. This finding opened GFP application to the analysis of hybridization and introgression status in transgenic crops.

4.3.1.5 Reef Coral Proteins

Biotechnological advances with AvGFP reporter gene have increased the demand of florescent proteins with different emission colors. Scientists have been successful in cloning *AsRed*, *AmCyan*, *ZsYellow*, *ZsGreen,* and *DsRed* genes from reef corals. The first report of the expression and characterization of one of these reef coral proteins as a marker in plants was DsRed (Jach et al. 2001) isolated from the reef coral *Discosoma* sp. Unlike *A. victoria* GFP, which is a monomer, reef coral florescent proteins are homotetramers that limit their application as fusion tag. Some of the first anthozoan fluorescent proteins were reported by Matz et al. (1999, 2002). Following these reports, Wenck et al. (2003) demonstrated that AsRed, AmCyan, ZsYellow, ZsGreen, and DsRed could be expressed and visualized in several monocotyledonous and dicotyledonous plants in both transient and stable gene integration. The AmCyan1, AsRed, and DsRed transgenic callus appeared to be yellow-green and red under white light. This is significant because, unlike GFP that require low wave length (UV) light source, AmCyan1, AsRed, and DsRed provided passive altering of tissues under room light (Wenck et al. 2003).

Many plants contain phenolic compounds that emit green fluorescence under UV light, used for GFP excitation and mimic GFP results (Stewart 2006). In this context, red fluorescent proteins gain an interest in plant applications because red fluorescence is rarely observed when higher wave lengths' light source (used red fluorescent proteins) is used for analysis of plant samples. As chlorophyll does not autofluoresce at higher wavelengths used for either AsRed or DsRed, these systems may be the best choice for utilization in plant biology.

One limitation of AsRed and DsRed is that in the native form both the proteins are found as tetramers, so they cannot be utilized as fusion tags in localization studies. Recently a monomeric form of DsRed has been produced (Merzlyak et al. 2007). Red fluorescent protein DsReD and its variant DsReD2 from reef corals have been used in transient assay and under stable nuclear genome integration in tobacco and soybean (*Glycine max*), respectively (Jach et al. 2001; Nishizawa et al. 2006). Fertile transgenic plants were regenerated without any negative morphogenic or physiological effect. In confirmation with Wenck et al. (2003) transgenic plants were distinguishable from nontransgenics under white light and worked as a visual marker for transgene expression.

Another red fluorescent protein, eqFP611, has been isolated from the sea anemone (*Entacmaea quadricolor*). Forner and Binder (2007) reported that transient and stable expression of eqFP611 protein had no detrimental effects on cell viability. Targeting of eqFP611 protein to mitochondria inherited mitochondria florescence. Another application of eqFP611 was its compatibility with GFP in dual labeling of plant cell organelles.

4.3.1.6 Oxalate Oxidases

The oxalate oxidases (OxO), which belong to the family of the germin-like proteins, catalyze the oxidation of oxalate, whereby hydrogen peroxide is formed. OxO enzymes are absent in most of the dicotyledonous plants and have a narrow window of expression in monocots (Grzelczak et al. 1985; Caliskan and Cuming 1998). Inexpensive substrates and availability of detection and quantification protocols (Thompson et al. 1995; Zhang et al. 1996) further add to the suitability of *OxO* as a reporter gene for plants (Simmonds et al. 2004). Activity of OxO can be measured histochemically as well as quantitatively (Simmonds et al. 2004) (Fig. 4.1c).

4.3.1.7 Anthocyanin Formation (Maize *R*, *C1,* and *B* Transcription Factors)

Maize *R*, *C1*, *P1,* and *B* transcription factor genes regulate the anthocyanin biosynthesis pathway in a tissue-specific manner in maize. The introduction of *C1*, R, and *B* regulatory genes under the control of constitutive promoters induces cell-autonomous anthocyanin pigmentation and allows for direct visualization of transformed cells and tissues (Ludwig et al. 1990; Radicella et al. 1992). Screening of transgenic tissue on the basis of anthocyanin pigmentation is a reporter system that would not require the application of selection pressure or external substrates for the detection of transgenic cells. Introduction of a plasmid encoding the maize *R* and *C*1 transcriptional factors, each under the control of a separate CaMV 35S promoter with maize *Adh1* intron, into immature wheat embryos resulted in the production of anthocyanin expressing cells. Pigmented cells were observed in the callus derived from these embryos for up to 1 month after bombardment, but these cells failed to proliferate (McKinnon et al. 1996). In a similar study, in which suspension cells of wheat were cotransformed with an anthocyanin marker and a selectable marker,

anthocyanin expressing callus was isolated (Dhir et al. 1994). Toxicity of these genes toward transformed cells and the requirement of environmental factors for expression (Chawla et al. 1999) limit the use of these genes as a reporter system.

4.3.1.8 Phytoene Synthase

Genes involved in carotenoid biosynthesis can be used as visual markers for identification of transgenic cells. The phytoene synthase enzyme catalyzes a reaction to produce phytoene from geranylgeranyl pyrophosphate. In the carotenoid biosynthesis pathway, phytoene is a precursor of the red carotenoid lycopene, a carotenoid that gives tomato fruit red color, followed by β-carotene in the next reaction. Trulson et al. (1997) reported that expression of phytoene synthase (from *Erwinia herbicola*) under transcription regulation of tomato callus-specific E8 promoter resulted in orange pigmentation in the callus. E8-phytoene synthase transgenic tomatoes were phenotypically similar to nontransgenic tomato plants, with the only difference being that fruits of transgenic plants developed color earlier than nontransgenic plants. Although phytoene synthase seems to be a good visual marker, its general application in other transgenic systems has been hampered because the other genes required for carotenoid synthesis are not present in all other plant species.

4.3.1.9 NPTII

NPTII, the most commonly used selectable gene, from transposon 5 (Tn5) of *E. coli* K12, encodes aminoglycoside 3-phosphotransferase II (APHII), commonly known as neomycin phosphotransferase II (NPTII, E.C 2.7.1.95), which inactivates the sugar-containing antibiotics, neomycin, kanamycin, geneticin (G418), and paromomycin by phosphorylation. Endogenous NPTII activity is rarely observed in plant tissues, making it a suitable reporter gene for plant applications. For an enzyme assay of NPTII, protein sample is first fractionated using nondenaturing polyacrylamide gel electrophoresis (PAGE), followed by phosphorylation of kanamycin with radioactively labeled ATP (^{32}P), by layering kanamycin containing agar over the enzyme containing polyacrylamide gel. The whole set is incubated at 35°C and the phosphorylation leading to incorporation of ^{32}P in kanamycin is detected by autoradiography (Fregien and Davidson 1985). Alternatively, filters with dot blots of the protein sample can be incubated with the substrates and then subjected to autoradiography. Presence of *NPTII* gene product can also be easily quantified by using NPTII-specific antibodies in an ELISA test (Nagel et al. 1992.)

4.3.1.10 Opines

The presence of opines in tumors in plants was discovered long before the identification of pathogenic Ti (tumor inducing) and Ri (root inducing) plasmids of

Agrobacterium or the demonstration of T-DNA transfer (Johnson et al. 1974). An advantage of opines as screenable markers for plant transformation is that they are natural markers of genetic transformation (crown gall or hairy root cells). The presence of opines in any plant material clearly indicates the transformed status of the plant cells. As a result, opine synthesis and the related genes have been widely used to construct numerous *Agrobacterium*-based vectors designed to engineer plant cells. Most of these vectors carry a wild-type or a modified *nos* (nopaline synthase) or *ocs* (octopine synthase) genes.

Nopaline and octopine are generally detected in plant extracts by high-voltage paper electrophoresis followed by reaction with phenanthrenequinone. Presence of UV-fluorescent products indicates the presence of opines. Yang et al. (1987) introduced a heat treatment step, compatible with paper electrophoresis that results in rapid production of a red-purple pigment. This colorimetric assay is sensitive to 1.25-μg quantities of opine and eliminates problems of background fluorescence encountered with crude plant extract.

4.3.1.11 Chloramphenicol Acetyl Transferase

Chloramphenicol acetyltransferase (CAT, E.C. 2.3.1.2), from the *E. coli Tn9* gene, neutralizes the antibiotic chloramphenicol by transfers of acetyl groups and thus changes its structure and prevents the antibiotic from inhibiting protein synthesis. CAT was the first bacterial gene to be introduced in plant cells (Herrera-Estrella et al. 1984), and it is still widely used as a reporter gene today, because of the stability of the enzyme and high sensitivity and ease of the enzymatic assay. The *CAT* gene is absent in mammals and higher plants, so its activity can be measured in the plant extract without any electrophoretic separation.

To measure the CAT activity, extracts from CAT transgenic plants are incubated with radiolabeled chloramphenicol. The acetylated products generated by the action of CAT are separated from the unmodified chloramphenicol by thin-layer chromatography and quantified by scraping the spots from the thin-layer plates and counting them by scintillation spectroscopy. Another assay substitutes the standard acetyl donor with butyl-CoA; the higher hydrophobicity of the butyl-chloramphenicol allowing for good separation of the butyl-compound and easy quantification of a large number of samples. Some plants have a nonspecific acetylase that can acetylate chloramphenicol as well, that necessitate use of controls. Extracts of some plant species may contain CAT enzyme inhibitors, although this problem can be eliminated in most cases with a heat treatment at 65°C.

4.3.2 PCR-Based Screening

The polymerase chain reaction (PCR) method is most sensitive among all molecular biology techniques used for testing the presence of the specific DNA sequence of a gene. Because of its sensitivity, the presence of a small quantity of contaminating

DNA can show false positives. For the screening of transgenic plants, PCR is generally performed with primers specific to the selectable marker and gene of interest, used for developing the transgenic plants. When large numbers of transgenic plants are screened, isolation of DNA becomes labor- and time intensive. As the PCR reaction does not require highly purified DNA, protocols have been standardized for rapid DNA isolation without freezing the plant samples in liquid nitrogen. A duplex PCR reaction is performed using two set of primers – one for the gene of interest (transgene or marker gene) and another for an enodgenous plant gene to confirm the DNA quality for PCR amplification (Mannerlof and Tenning 1997; Xu et al. 2005).

The advent of real-time PCR has made multiplexing and relative quantification easy. As compared with traditional transgene copy number detection technologies such as Southern blot analysis, real-time PCR provides a fast, inexpensive, and high-throughput alternative. Real-time PCR can be used to determine copy number and zygosity in transgenic plants (as reviewed by Bubner and Baldwin 2004; Prior et al. 2006; Yuan et al. 2007). The availability of different chemistries for fluorescence detection in real-time PCR created some confusion about their relative merits. In a comparative study, molecular beacon, SYBR Green, TaqMan, and MGB assays were designed for the event-specific detection and quantification of the 3' integration junction of GTS 40-3-2 (Roundup Ready) soybean. Sensitivity as well as robustness in the presence of background DNA was tested. None of the PCR-based approaches appeared to be significantly better than any of the other (Andersen et al. 2006). In another study, five different chemistries employing TaqMan, Lux, Plexor, Cycling Probe Technology, and LNA were tested, and it was concluded that none of chemistries outperformed the others (Gasparic et al. 2008).

Adaptor ligation PCR (AL-PCR) of genomic DNA flanking T-DNA borders is another attractive alternative to genomic DNA blot hybridization. An adapter is ligated to restriction enzyme-digested genomic DNA and PCR is carried out using primers specific for a T-DNA border and the adapter sequence. Each independent integration event shows a PCR amplicon of different size (Spertini et al. 1999). Alternatively PCR amplicons can be sequenced to find the locus of integration. The AL-PCR patterns obtained in *Allium cepa* were specific and reproducible for a given transgenic line and gave insight in the number of T-DNA copies (Zheng et al. 2001).

The presence of latent *Agrobacterium* in the plant can give false-positive results in a PCR analysis of putative transgenic plants. Nain et al. (2005) have reported a simple protocol for PCR analysis of *Agrobacterium*-contaminated transgenic plants that is based on denaturation and renaturation of DNA in a time-dependant manner. The contaminating plasmid vector becomes double stranded most quickly during renaturation and is cut by a restriction enzyme having site(s) within the PCR amplicon. Once this plasmid DNA is digested, it will eliminate PCR amplification from contaminating plasmid DNA. The genomic DNA with a few copies of the transgene remains single stranded and unaffected by the restriction enzyme, leading to amplification by PCR. Hence, only the transgene present in the genomic DNA is amplified by PCR (Fig. 4.2).

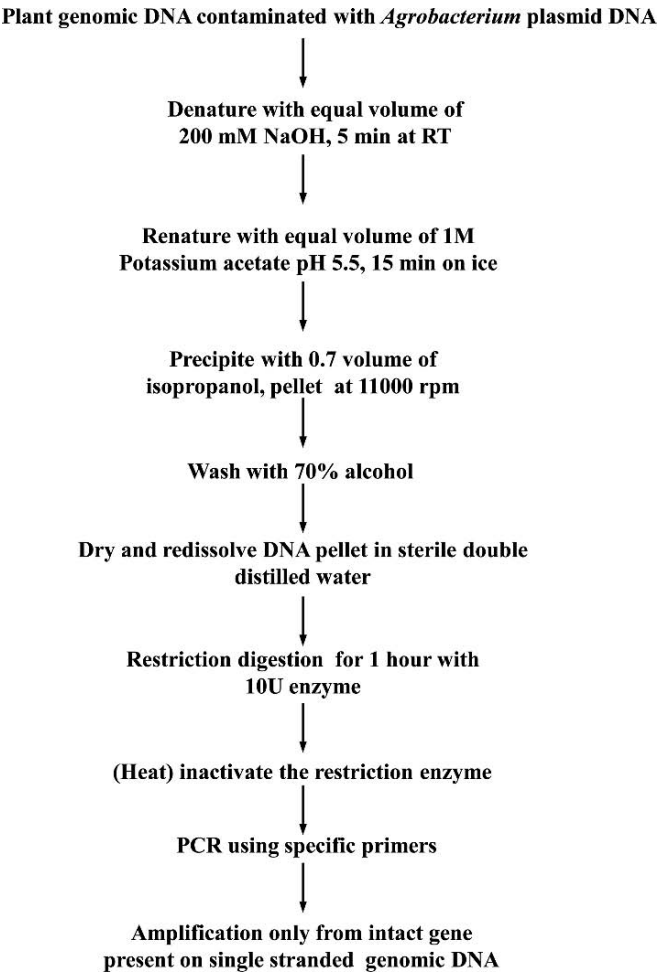

Fig. 4.2 Confirmation of transgene integration in plant genome by PCR. After processing the genomic DNA from putative transgenic plants, PCR amplification comes only from transgene integrated in plant genome Nain et al. (2005)

4.3.3 Southern Hybridization Analysis

Southern blotting (Southern 1975) is a technique for transfer of single-stranded (denatured) DNA molecules from an electrophoresis gel to a nitrocellulose or nylon membrane. The nitrocellulose membrane is incubated with a specific radiolabeled probe and the location of the DNA fragment that hybridizes with the probe is detected by autoradiography (Sambrook et al. 1989) or with a Phosphor Imager (for Quantitative Filmless Autoradiography). High sensitivity, low background, and

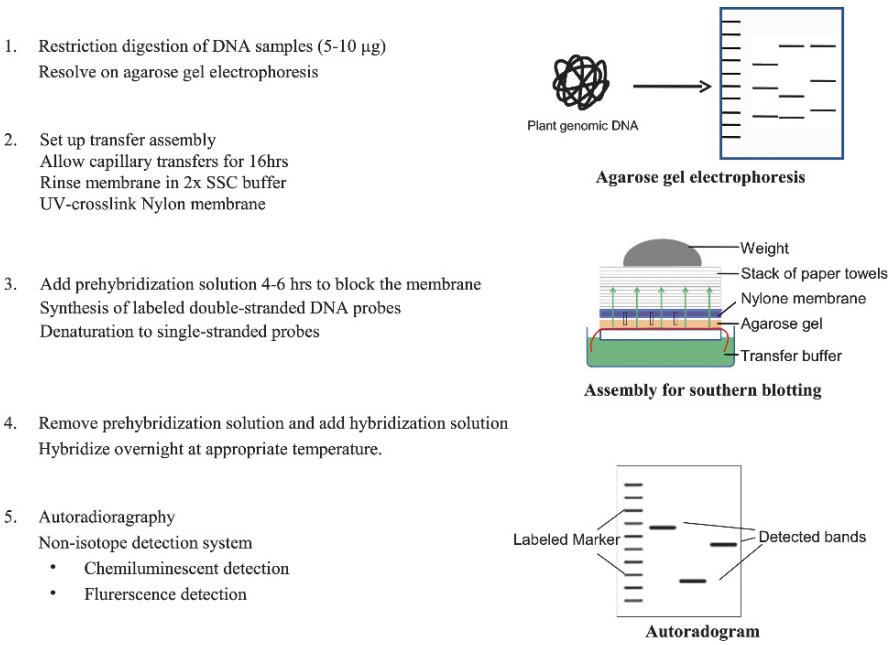

Fig. 4.3 Methodology for confirmation of transgene integration in plant genome by Southern hybridization

determination of transgene copy number integrated to plant genome make this technique indispensable for molecular analysis of transgenic plants (Fig. 4.3).

The standard radioactively labeled DNA detection system used in such experiments requires licensing to handle radioactive materials. The development of a nonradioactive biotin, digoxigenin (DIG), and fluorescein isothiocyanate (FITC) labeling-based nucleic acid detection system has made such DNA hybridization experiments safe and more generally feasible (Leary et al. 1983; Peterhaensel et al. 2007). Bound biotin labeled probe is detected by an alkaline phosphatase conjugate of streptavidin, while for digoxigenin detection an antidigoxigenin antibody conjugate is used. Alkaline phosphatase conjugate is visualized with chromogenic alkaline phosphatase substrates. Chromogenic substrates produce a colored signal directly on the membrane. More sensitive chemiluminescence substrates for alkaline phosphatase produce light that can be conveniently recorded with X-ray film (as with ^{32}P probes). Fluorescence signal emitted by FITC labeled probe is recorded with chemiluminescence detection system. Although various modifications of Southern hybridization have been reported, the use of radiolabeled probes is still the most common and the most reliable method.

When determining the integration of transgenes into the plant genome, Southern hybridization is the most reliable technique. Sometimes reporter gene signal may be absent even after the integration of transgene in the plant genome. This may be because of silencing of the transgene after integration, low RNA stability, poor translation efficiency or integration of a truncated gene. Under these conditions

only Southern hybridization can give a true picture of an integration event. Furthermore, Southern hybridization can reveal the presence of genomic DNA flanking the transgene, which is the ultimate confirmation of insertion, and which is not always possible using PCR. However, Southern hybridization requires a large quantity (5 μg) of DNA from each transgenic plant that can limit its application when plant tissue is not available in adequate quantity.

To determine the transgene copy number and distinguish different integration events, the plant genomic DNA is digested with a restriction enzyme that does not have a restriction site in the T-DNA and when the T-DNA or transgene is used as a probe, each independent integration event will appear as a unique band. Presence of more than one band will indicate a multicopy transgenic event. If the restriction enzyme used for genomic DNA digestion has a unique site between the marker gene and gene of interest (transgene) and the membrane is probed with the marker gene and the transgene separately, then each integration event will show up as a single band. The size of the band identified with each probe will be different, but the number of bands in each plant sample will remain the same. Any mismatch will represent an integration of a truncated copy. To identify a multiple-copy tandem integration at one locus, genomic DNA is digested with restriction enzymes flanking the gene of interest in the T-DNA. Probing the blot with the transgene will give a single band of size of the transgene in all the samples, but intensity of the band will be proportionate to the number of gene copies integrated (Bhat and Srinivasan 2002).

4.3.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) tests for the presence of the specific protein that the transgene produces in the plant. ELISA procedures use antibodies that react specific with the new protein(s) produced in the transgenic plants. There are different versions of the ELISA method used for detection of heterologous protein expressed in transgenic plants. One method uses lateral flow strips that deliver results in 2–5 min. This "strip test" technology is also marketed as the "dipstick" procedure. Advantages of the ELISA strip tests are speed, relative ease of use, and low cost. On the other hand, a major disadvantage is that it cannot quantify the protein of interest in a transgenic plant sample. Another version of the ELISA test, the "plate test," allows quantification of protein of interest. A standard ELISA plate can test 96 samples at a time, including positive and negative controls. Intensity of color indicates the amount of the protein present (Fig. 4.4). The plate test can take 2–4 h and is more laborious and costly than the strip test.

4.4 Marker-Removal Strategies

Marker genes play a crucial role during plant transformation for identifying rare transgenic cells/plants. However, the presence of marker DNA sequences in the final transgenic plants is often problematic for commercial biotechnology products

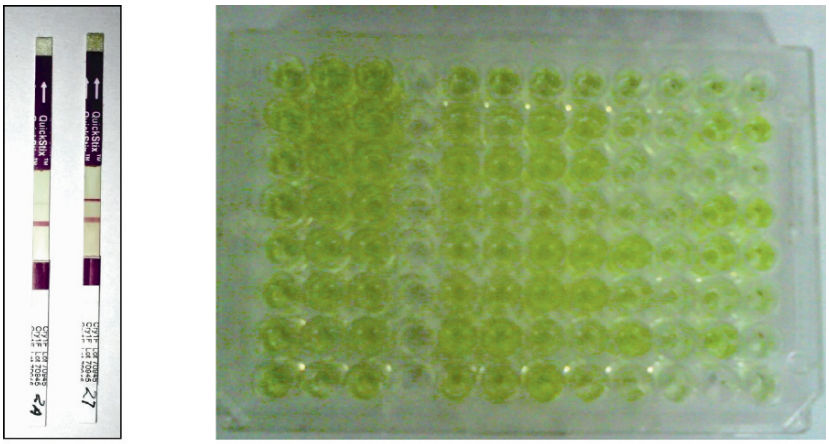

Fig. 4.4 Confirmation of transgene expression by strip test and ELISA plate method (V Nain, unpublished)

because of biosafety regulatory requirements and public concerns, especially when marker genes of nonplant origin are employed. Additionally, elimination of marker genes, whose functions are no longer in need, can save the cell from the burden of maintaining the unwanted transgene, allow gene stacking through reuse of the same marker gene in subsequent transformations, and avoid negative pleiotropic effects that may be associated with the marker gene in plants. These concerns have prompted the development of several approaches to generate marker-free transgenic crops. Technologies in this area have advanced greatly in recent years and new marker-removal strategies are expected to be continually explored. Currently, the use of site-specific-recombinases under the control of inducible promoter presents the greatest promise in terms of efficiency, preciseness, and time period required.

4.4.1 Cotransformation and Subsequent Segregation

The simplest strategy to eliminate marker gene is the cotransformation of genes of interest with marker genes followed by segregation through sexual crosses. Both desirable transgene and marker gene can be delivered into plant genomes by separate plasmids in one or two *Agrobacterium* strains or with single plasmids carrying multiple T-DNA regions (transposon-based), based on *Agrobacterium*- or biolistics-mediated transformation (Miki and McHugh 2004; Zhao et al. 2007). The unlinked marker gene can subsequently be removed from the plant genome during segregation and recombination that occurs during sexual reproduction by selecting on the transgene of interest, and not the marker gene, in progeny. Screening for the

Table 4.9 Chemicals and enzymes for the conditional-negative selection of transgenic tissues[a]

Substrates	Genes	Enzymes	Sources	Genome	References
5-Fluorocytosine	*codA*	Cytosine deaminase	*Escherichia coli*	Nuclear, plastid	Stougaard (1993); Serino and Maliga (1997)
Naphthalene acetamide	*aux2*	Amido hydrolase	*Agrobacterium rhizogenes*	Nuclear	Béclin et al. (1993)
Indole-3-acetamide	*tms 2*	Indoleacetic acid hydrolase	*Agrobacterium tumefaciens*		Depicker et al. (1988)
Dihaloalkanes	*dhlA*	Dehalogenase	*Xanthobacter autotrophicus*	Nuclear	Naested et al. (1999), Moore and Srivastava (2008)
Sulfonylurea R7402	*cyp105a*	Cytochrome P450 mono-oxygenase	*Streptomyces griseolus*	Nuclear	O'Keefe et al. (1994)
Allyl alcohol	*cue*	Alcohol dehydrogenase	*Arabidopsis thaliana*	Nuclear	Lopez-Juez et al. (1998)
ganciclovir	*HSVtk*	thymidine kinase type 1 gene	*Homo sapiens* virus	nuclear	Czakó et al. (1995)
glyceryl glyphosate	*pehA*	phosphonate monoesterase	*Burkholderia caryophilli PG2982*	Nuclear	Dotson et al. (1996)

[a]Table was adapted from Miki and McHugh (2004)

progeny without the marker gene can be assisted by PCR or Southern hybridization analysis, as well as by incorporating an extra conditionally negative or scorable marker gene next to the original selective marker gene in the same construct (Darbani et al. 2007). Some selective marker genes have been developed for this purpose (Table 4.9). Each of these selective markers encodes an enzyme that converts nontoxic agents to toxic agents resulting in the death of the transformed cells containing the marker genes used for transformation selection or screening.

Recently, a novel marker gene, *dao1* from yeast, has been established for either positive or negative marker in *Arabidopsis*, depending on the substrate (Erikson et al. 2004). D-amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the oxidative deamination of a range of D-amino acids. This enzyme can metabolize toxic D-alanine and D-serine into nontoxic products, whereas it converts D-isoleucine and D-valine, which have low toxicity, into the toxic keto acids 3-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate, respectively. Hence, both positive and negative selection is possible with the same marker gene through changing D-alanine or D-serine to D-isoleucine or D-valine for the substrates. The bifunctional *dao1* marker gene yielded unambiguous results and allowed selection immediately after germination in *Arabidopsis* (Erikson et al. 2004).

The removal of selectable markers by cotransformation can be time consuming and labor intensive. The requirement of sexual crosses limits its application in woody, vegetatively propagated, or sterile plant species or cultivars. The efficiency of this strategy also depends on the loose linkage between the cointegrated DNAs.

4.4.2 Transposon-based Marker Gene Removal

The maize *Ac*/*Ds* transposable element system has been used to transpose marker genes and transposases from the T-DNA, thus leaving only the gene of interest in the inserted copy of the T-DNA (Yoder and Goldsbrough 1994). In one such system, the maize *Ac* transposable element was engineered to contain the *ipt* gene, which conferred an extreme shooty phenotype in the first positive selective step and also served as a negative selectable marker in the second selection step (due to the elimination of the *ipt* and transposase genes by transposition, normal shoots appeared after several weeks or months in culture) (Ebinuma et al. 1997). A marker-free frequency of about 5% was obtained in tobacco and hybrid aspen (Ebinuma et al. 1997). Since this system does not require a sexual reproduction step, it is an alternative for vegetatively propagated cultivars and plants with a long reproductive cycle. However, several drawbacks of this system, such as imprecise excision, low excision frequency, and genomic instability of transgenic plants, have undermined its efficacy (Scutt et al. 2002; Darbani et al. 2007). The maize *Ac*/*Ds* transposable element system has also been used to transpose (separate) the gene of interest that is previously linked together with marker gene and transposase in the T-DNA. However, this technology relies on sexual segregation to remove the marker gene and the transposase (Miki and McHugh 2004).

4.4.3 Site-specific Recombination-mediated Marker Deletion

The site-specific recombination systems mediate control of a variety of biological functions by carrying out precise excision, inversion, or integration of defined DNA units in their natural prokaryote and lower eukaryote hosts. Due to their accuracy and relative simplicity, the Cre/*lox* genes of bacteriophage P1 of *E. coli*, R/*RS* from the SR1 plasmid of *Zygosaccharomyces rouxii*, and FLP/*FRT* from the 2-μm plasmid of *Saccharomyces cerevisiae* have been the focus of the most intense studies in plants and other organisms. These systems function through the interactions of a single recombinase (e.g., Cre, R, FLP) with a pair identical recognition target sites (34 bp *lox* and *FRT*; 31 bp *RS*) in a "cut and paste" recombination process. The required recombinase can be introduced to target sites in transformants by retransformation to activate the marker gene excision. The recombinase locus is then removed by sexual segregation (see for review Miki and McHugh 2004; Darbani et al. 2007; Gidoni et al. 2008). Since constitutive expression of recombinase may cause aberrant developmental phenotypes in plants, the use of site-specific recombinases under the control of inducible promoters provides a more promising avenue. Table 4.10 lists the inducible promoters that have been paired with recombinase genes for marker gene excision. Depending on the type of inducible promoters being utilized, this inducible recombination strategy can provide further control

Table 4.10 Induced recombination-mediated auto-excision of markers and recombinase genes

Induction signal/ promoter system	Recombination system/plant tested	Stage of induction/ treatment	Excision rate	References
Chemical/ herbicide antidote-inducible maize GST	R-RS/tobacco	Shoot explants/ inducer in the medium	14–58%[a]	Sugita et al. (2000); Ebinuma et al. (2001)
Chemical/β-estradiol inducible transactivator XVE	Cre-lox/*Arabidopsis*, rice, tomato	Shoot explants, callus-germinating seeds/inducer in the medium	15–66%[a,b,c]	Zuo et al. (2001); Sreekala et al. (2005); Zhang et al. (2006)
Chemical/ dexamethasone-activated R-LBD	R-RS/strawberry	Regenerating leaf explants/ inducer in the medium	N.D.	Schaart et al. (2004)
Chemical/Salicylic acid-induced activated PR-1a	Cre–loxP/tomato	Shoot explants/ inducer in the medium	38.7%[a]	Ma et al. (2009)
Heat-shock/ *Arabidopsis* HSP81–1	Cre-lox/*Arabidopsis*	Whole seedlings/ ×2 alternate 37°C-16 h and recovery	N.D.	Hoff et al. (2001)
Heat-shock/ *Arabidopsis* HSP81–1	Cre-lox/tobacco	Whole seedlings/ ×2 alternate 37°C-16 h and recovery	< 100%[b]	Liu et al. (2005)
Heat-shock/ soybean HSP17.5E	Cre-lox/maize	Callus and immature embryos/42°C for 3–5 h	< 100%[b,c]	Zhang et al. (2003)
Heat-shock/ soybean HSP17.5E	Cre-lox/tobacco	Seed, leaf/×3 alternate 42°C-2 h and recovery	40–80%[a,b,c]	Wang et al. (2005)
Heat-shock/ *Drosophila* hsp70	Cre-lox/potato	Shoot internodes and mini-tubers/ 42°C for 2–3 h	5–14%[a]	Cuellar et al. (2006)
Heat-shock/ *Arabidopsis* HSP18.2	FLP-loxP, FRT/ tobacco	Regenerating leaf explants/65°C for at least 1 h	60%[a]	Luo et al. (2008)
Embryo-specific/ *Arabidopsis* app1	Cre-lox/soybean	Somatic embryogenesis	5–59%[a,b,c]	Li et al. (2007)
Microspore-specific/tobacco NTM19	Cre-lox/*Arabidopsis*, tobacco	Early pollen development	< 99.98%[a,c]	Mlynárová et al. (2006)
Pollen-specific/*B. campestris*	Cre-or FLP-lox-FRT fusion/tobacco	Mature pollen development	< 100%[a,c]	Luo et al. (2007)

(*continued*)

Table 4.10 (continued)

Induction signal/ promoter system	Recombination system/plant tested	Stage of induction/ treatment	Excision rate	References
BGP1 and tomato LAT5				
Pollen and seed-specific/ *Arabidopsis* PAB5		Pollen and seed development		
Male germline-specific/ *Arabidopsis* SDS	Cre-lox/*Arabidopsis*	Male gamete development	83–100%[a,c]	Verweire et al. (2007)
Floral meristem-specific/ *Arabidopsis* AP1		Flower meristem-male and female germ lines		
Floral-specific/rice OsMADS45	Cre-lox/rice	Floral organs	13–100%[a,c]	Bai et al. (2008)

GST Glutathione-S-transferase (GST-II-27) promoter-MAT vector, *R-LBD* glucocorticoid receptor ligand binding domain fused to the C-terminus of R recombinase gene, *AP1* Apetala1, *SDS* solo dancers, *N.D.* not determined

Table was adapted from Gidoni et al. (2008)

[a]Complete plant gene excision events

[b]Incomplete plant (chimeric) gene excision events

[c]Provided indications for germline excision events

of the excision process on timing, flexibility and tissue specificity. In addition, this inducible system allows a single recombination construct where a marker gene and a recombinase gene coreside between the recombination target sites; therefore, genetic segregation is not required to remove the recombinase gene since excised circular DNA containing both marker and recombinase genes are expected to be lost via cellular degradation. Transient expression of recombinase through cocultivated or infiltration of *Agrobacterium* T-DNA vectors or systemic infection with plant virus vector has also been developed for tobacco (Gleave et al. 1999; Kopertekh and Schiemann 2004; Jia et al. 2006) and maize (Kerbach et al. 2005). Employment of two site-specific recombination systems has been recently explored in tobacco (Luo et al. 2007) and maize (Djukanovic et al. 2008). It was found that the two site-specific recombination systems could provide more efficient and complete excision. In addition to deletion of a marker gene from the transgenic plant, site-specific recombination systems can also be useful in site-specific integration of a gene into a predetermined genomic location in a precise, single copy pattern.

Besides the Cre/*lox*, R/*RS*, and FLP/*FRT* mentioned earlier, new recombination systems are being discovered and explored, including the β/six from *Streptococcus pyogenes* (Grønlund et al. 2007) and a small serine resolvase ParA from bacterial plasmids RK2 and RP4 catalyses (Thomson et al. 2009), which have proven efficient in marker gene deletion in tobacco and *Arabidopsis*. Site-specific recombination holds great promise for marker deletion and has advanced quickly in recent

years. However, the commercial potential of this technology has not been yet demonstrated.

4.4.4 Intrachromosomal Homologous Recombination System

The intrachromosomal homologous recombination system is very similar to the site-specific recombination systems described above, except that there is no requirement for recombinase, whose action on cryptic excision sites in the plant genomes may cause pleiotropic effects. When placed between two 352 bp attachment P (attP) regions in bacteriophage, three marker genes (*nptII*, *gfp*, and *tms2*) were deleted from transgenic tobacco with a deletion frequency of 44% (Zubko et al. 2000). Interestingly, most plantlets that have lost the marker gene region also have lost transgene regions outside the attP cassette, suggesting that intrachromosomal homologous recombination is not always associated with precise homologous recombination between the two attP regions but that it can generate larger deletions probably as a result of illegitimate recombination (Zubko et al. 2000). The attP intrachromosomal excision system has also been utilized in transformation of tobacco plastids (Kittiwongwattana et al. 2007).

4.4.5 Cytokinin-Based Backbone-Free Approach

Recently, Richael et al. (2008) have described a new method that was based on transient expression of the bacterial isopentenyltransferase (*ipt*) gene that was positioned within the backbone (outside the T-DNA region) of binary vectors. It was found that the resulting temporary production of the natural cytokinin isopentenyl adenosine induced explants to produce shoots on media containing neither a selection agent nor synthetic hormones. This approach has been tested in various Solanaceous plant species including potato, tomato, tomatillo, and tobacco, as well as canola (Richael et al. 2008; Rommens et al. 2008). Transformation frequencies achieved were similar to conventional backbone-free transformation with marker-containing T-DNAs and higher than conventional methods that simply omit a selection step (de Vetten et al. 2003). Since shoots displaying a cytokinin overexpression phenotype were ignored and only shoots with a wild-type appearance were selected, the abnormal shoot morphology usually associated with the *ipt* gene in transformants is not an issue in this approach. This approach provides both marker-free and backbone-free transformation.

4.4.6 Radiation Method

The use of γ-radiation to physically remove a marker gene previously introduced into the soybean genome was evaluated by Tinoco et al. (2006). Preliminary data

indicated a very low success rate of marker gene removal. In most cases, the gene of interest was deleted along with the marker gene. In addition, abnormal phenotypes were observed

4.5 Conclusions

Selectable and scorable marker genes play a vital role in identifying transformed plant cells. A wide range of marker genes have been employed for successful plant genetic transformations. Due to the substantial public concern about the potential spread of marker genes of nonplant origin, there is a momentum for research toward environment-friendly selection system involving natural plant materials and precise marker gene removal.

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Chapter 5
Levels and Stability of Expression of Transgenes

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5.1 Introduction

It is well known that in a given cell, at a particular time, only a fraction of the entire genome is expressed. Expression of a gene, nuclear, or organellar starts with the onset of transcription and ends in the synthesis of the functional protein. The regulation of gene expression is a complex process that requires the coordinated activity of different proteins and nucleic acids that ultimately determine whether a gene is transcribed, and if transcribed, whether it results in the production of a protein that develops a phenotype. The same also holds true for transgenic crops, which lie at the very core of insert design.

There are multiple checkpoints at which the expression of a gene can be regulated and controlled. Much of the emphasis of studies related to gene expression has been on regulation of gene transcription, and a number of methods are used to effect the control of gene expression. Controlling transgene expression for a commercially valuable trait is necessary to capture its value. Many gene functions are either lethal or produce severe deformity (resulting in loss of value) if overexpressed. Thus, expression of a transgene at a particular site or in response to a particular elicitor is always desirable.

Usually, the regions responsible for the initiation of transcription lie within the 5′ region, upstream to the coding sequence of the gene. These are the promoter regions, defined as *cis*-acting (as they are on the same DNA strand that codes for the gene) nontranscribed elements, which provide sequences for the binding of various transcription initiation factors and RNA polymerase.

One can use a promoter that has known regulatory characteristics; for example, a promoter that is expressed throughout the plant tissue or only in vascular tissues, in

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the leaf epidermis, seed endosperm or embryo, and so on. One can mix and match fragments of DNA and transcription factors to develop chimeric promoters that have the desired patterns and levels of gene expression.

In this chapter, we will discuss the basic aspects of designing genes for insertion and quantifying transgene expression, followed by the different types of promoters and their use in transgenic crops. Also, several factors responsible for high-level expression and stability in transgenic plants/crops will be discussed.

5.2 Gene Design for Insertion

Once a gene of choice has been targeted and cloned, it has to undergo several modifications before it can be effectively inserted into a plant. A promoter sequence is added for the gene to be expressed. Most promoters used for transgenic crop varieties have been "constitutive," i.e., causing gene expression throughout the life cycle of the plant in most of the tissues. The most commonly used constitutive promoter is CaMV 35S, from the cauliflower mosaic virus, which generally results in a high level of expression in most plants. Some promoters are more specific and are discussed in detail in Sect. 5.4.

Genes of interest are sometimes modified to achieve high level of expression. As plants prefer G-C rich regions, as compared to A-T rich bacterial genes, in order to overexpress bacterial genes in plants, A-T rich regions are to be substituted by G-C rich regions in such a way that the amino acid sequence of the protein remains unaltered (Evans et al. 2003). A selectable marker gene is inserted into the construct so as to identify the cells or tissues that have been successfully transformed (as discussed in Chap. 4). In some cases (e.g., resistance to pesticides), the transgene itself acts as a selectable marker. In other instances, a reporter gene is also inserted in the construct. A reporter gene is a coding sequence that upon expression in the transgenic plant provides conclusive evidence of genetic transformation. These reporter genes are very useful for transient expression experiments where the spatial and temporal activity of a promoter can be elucidated. The genes naturally exhibit an enzyme activity that does not exist in the host plant. Most common reporter genes are from bacteria, insects, or jellyfish as these organisms are so unrelated to angiosperms that their *cis*-regulatory elements are not functional in plants. Thus, when cloned into plant transformation vectors, a terminator sequence should also be fused downstream to the gene. Commonly used reporter genes are *CAT* (*E. coli*), β-*GUS* (*E. coli*), luciferase (firefly), green fluorescence protein (jellyfish), etc.

5.3 Quantification of Transgene Expression

It is necessary to know how a transgene is expressing in order to evaluate its effectiveness and level of expression in transgenic plants. The transgene copy number can greatly influence the expression level and genetic stability in the

plant, and therefore, estimation of the transgene number is of prime importance (Bhat and Srinivasan 2002). Previously, Southern and Northern analyses were used for this purpose (Sabelli and Shewry 1995a, b). But, with time, other methods such as comparative genomic hybridization, fluorescence *in situ* hybridization, multiplex amplifiable probe hybridization, and microarray had been employed to determine the transgene copy number. All these methods are time consuming, laborious, and require large quantities of DNA. Moreover, nucleic acid hybridization-based techniques often involve the application of hazardous radioisotopes.

Recently, quantitative real-time PCR (qPCR) has proved to be an efficient method for transgene expression studies in plants. In qPCR, expression level is monitored per cycle of the reaction, comparing the fluorescent signal generated by the DNA or mRNA sample proportional to its initial quantity (Page and Minocha 2004). It has proved to be a more sensitive and rapid method, providing stringent evaluation through the use of SYBR Green I Fluorescent intercalating dye, which has the ability to detect a single gene among a number of genes in combination with highly specific gene primers. Till now, qPCR has been extensively applied to several transgenic crops such as maize (Ingham et al. 2001; Song et al. 2002; Shou et al. 2004; Assem and Hassan 2008), wheat (Li et al. 2004), rice (Yang et al. 2005), potato (Toplak et al. 2004), rapeseed (Weng et al 2004), tomato (Mason et al. 2001), tobacco (Miyamoto et al. 2000), cassava (Beltrán et al. 2009), and strawberry (Schaart et al. 2002) for analyzing transgene expression.

5.4 Promoters

As discussed earlier, the 5′ upstream regions of a gene are not transcribed but provide sites for attachment of transcription initiation factors. The promoter itself contains many elements (short regions of a defined DNA sequence) for initiation factor attachment. The very basic of these elements is the TATA box, which is present about 25–30 bp upstream of the transcription start site and is primarily responsible for the correct positioning of RNA polymerase II. Many genes contain multiple operational TATA boxes, for example, three for *inrpk1* gene in *Ipomoea nil* (Bassett et al. 2004) and three for *phas* gene in *Phaseolus vulgaris* (Grace et al. 2004). It was thought earlier that the absence of TATA box is associated with constitutively expressing housekeeping genes, but recently TATA box was found to be absent in some inducible genes as well. Apart from TATA box, CAAT and GC boxes are also found to be present upstream; they too enhance the activity of RNA polymerase. Sequence elements like TATA boxes are also referred to as minimal or core promoter elements.

Along with the core promoter elements, other sequence elements are also found that provide sites for attachment of specific transcription factors or enhancer binding protein that trigger transcription of the gene (Alberts et al. 2002). These sequence elements are also called regulatory elements, enhancer binding elements, or simply enhancers. Enhancers are consensus DNA sequence motifs and are

associated with levels, place, and timing of expression in response to internal or external (biotic or abiotic) factors. Enhancers can be located upstream, downstream, within coding regions or even in the intron sequences. One of the chief factors responsible for control of gene expression at the transcription level is the activation of enhancer sequences. A few *cis*-elements have the ability to silence or repress expression of the gene; these are called silencers. The activities of some of the plant promoters are summarized in Table 5.1. The table was generated using TGP, PlantCARE, NCBI, and Plant-Promoter databases for different genes specifically expressed in plants under the influence of suitable reporters/inducers, resulting in higher expression.

5.4.1 Types of Promoters and Their Applications in Transgenic Crops

5.4.1.1 Constitutive Promoters

Constitutive promoters maintain a constant level of activity. The cauliflower mosaic virus (CaMV) 35S promoter (derived from a DNA viral genome) is probably the most widely used plant promoter (Odell et al. 1985). Although "constitutive," many show differences in the level of expression in different tissues. Apart from delivering very high levels of expression in virtually all regions of the transgenic plant, the CaMV 35S promoter is easily obtainable for research purposes as plant transformation vector cassettes that allow for easy subcloning of the insert transgene of interest.

High levels of transgene expression can be achieved by the CaMV 35S promoter in both monocot and dicot plants (Benfey et al. 1990; Battraw and Hall 1990). The original full-size promoter (−941 to +9) has no significant difference in activity when compared to a −243 bp fragment. Interaction between the *cis*-acting elements within 343 bp upstream of the promoter results in high constitutive expression (Fang et al. 1989). However, tissue-specific individual elements have also been found (Benfey and Chua 1989). For the control of expression in specific tissues, two domains "Domain A" (−90 to +8) and "Domain B" (−343 to −90) are very important. Domain A is involved in expression in roots (Lam et al. 1989), while Domain B contains a conserved GATA motif, very much similar to the light responsive *cis* elements of light inducible promoters (Potenza et al. 2004). Even though CaMV 35S is a very strong promoter, it is strongly down-regulated in plant parasitic nematode feeding sites (Urwin et al. 1997). With the success of CaMV 35S promoter, other viral promoters have also been developed. They include the cassava vein mosaic virus (CsVMV; Verdaguer et al. 1996, 1998; Li et al. 2001), Australian banana streak virus (BSV; Schenk et al. 2001), mirabilis mosaic virus (MMV; Dey and Maiti 1999), and figwort mosaic virus (FMV; Maiti et al. 1997) promoters.

Table 5.1 Promoters used for transgene expression in plants

Promoter	*Gene*	Specificity	Reporter/inducer	Comment	References
At:SAG12_P1, P2	*sag12*, senescence-associated gene	transgenic *Arabidopsis* old leaves	mRNA, GUS activity/ auxin, cytokinin, sugar	Essential for senescence-specific regulation of sag12	Noh and Amasino (1999)
Ms:PEPC7_P1, P2	*pepc7, pepc*	transgenic alfalfa plants	GUS activity/Rhizobium	very high levels of GUS activity	Pathirana et al. (1997)
Nt:COMTII_P1, P2, P3	*comtII*	transgenic tobacco leaves	GUS activity/chitin, glucan, TMV, pectin, wounding, MeJa	two to seven-fold increase in GUS activity	Toquin et al. (2003)
Ib:BMY1_P1, P2	*bmy1, amyb, beta-amy*	transgenic tobacco plants	GUS activity/sucrose (10%)	20–57-fold higher GUS activity	Maeo et al. (2001)
Dc:HCBT2_P1 P2, P3, P4	*hcbt2*	transgenic parsley protoplasts	GUS activity/elicitior	3.7–5.5-fold higher GUS activity	Yang et al. (1998)
Le:E4_P1, P2	*e4*	transgenic tomato plants	GUS activity/Ethylene	Increased 10–22-fold in unripe and 1,000-fold in ripened fruit	Montgomery et al. (1993a)
Zm:SBE1_P1, P2	*sbe1*	transgenic maize suspension endosperm cells	LUC activity/sucrose (9%)	two-fold greater LUC activity	Kim and Guiltinan (1999)
Vf:GRP3_P1, P2, P3	*grp3, glycine-rich early nodulin*	transgenic *Vicia* hirsute (5 week-old) nodules	GUS activity/Rhizobium	Essential for full promoter activity	Kuster et al. (1995)
Nt:CHN48_P1, P2	*chn48*	transgenic tobacco calli	LUC activity/elicitor	10–40-fold higher activity	Yamamoto et al. (1999)
At:P5CSA_P1, P2	*P5CSA_P1*	transgenic *Arabidopsis* plants	GUS activity/dehydration	five-fold increase in activity	Yoshiba et al. (1999)
Ib:SPOA1_P1, P2	*spoa1, gspo-a1*	transgenic tobacco plants	GUS activity/sucrose (3%)	tissue specific GUS staining	Ohta et al. (1991)
Pc:CMPG1b_P1	*cmpg1, eli17*	transgenic parsley suspension cells	GUS activity/elicitor	44-fold higher activity	Kirsch et al. (2001)
Pc:PR2_P1, P2	*pr2*	transgenic parsley protoplasts	GUS activity/elicitor	three- to eight-fold higher activity	van de Löcht et al. (1990)

(*continued*)

Table 5.1 (continued)

Promoter	*Gene*	Specificity	Reporter/inducer	Comment	References
Pc:PR1.1_P1, P2, P3	*pr1.1*	transgenic parsley suspension cells	GUS activity/elicitor	7.5–12.1-fold higher activity	Rushton et al. (1996)
Pc:PR1.2_P1, P2	*pr1.2*	transgenic parsley suspension cells	GUS activity/elicitor	5.2–15.2-fold higher activity	Rushton et al. (1996)
Ps:PSL_P1, P2, P3	*psl*	transgenic tobacco seeds	GUS activity, protein	High level of expression	de Pater et al. (1996)
Lg:LHCB2_P1, P2, P3, P4, P5	*lhcb2, cabAB19*	transgenic swollen duckweed seedlings	LUC activity/red light (2 min)	2–14-fold higher activity	Kehoe et al. (1994)
Np:LHCB1.2_P1, P2, P3, P4, P5	*lhcb1.2*	transgenic tobacco seedlings, transgenic *Arabidopsis* seedlings	GUS activity/far-red light (cont.), red light (pulses 3 min)	Medium 8–20-fold higher expression level	Cerdan et al. (2000)
Os:Amy1A_P1, P2, P3, P4	*amy1A*	transgenic rice embryos	GUS activity/Glucose, gibberellin A3	two- to six-fold lower expression with glucose, higher with gibberellin A3	Morita et al. (1998)
Os:Amy3D_P1	*amy3D*	transgenic rice embryos	GUS activity/glucose	six-fold lower Expression	Morita et al. (1998)
Zm:SH1_P1	*sh1*	transgenic tobacco plants	GUS activity/anaerobic conditions	High level of expression	Yang and Russell (1990)
At:CAB3_P1, P2, P3, P4	*cab3*	transgenic tobacco shoots (3–4 week-old), suspension cells	CAT activity/white light	Medium to high level of expression	Mitra et al. (1989)
Pc:WRKY1_P1, P2, P3	*wrky1*	transgenic parsley protoplasts	GUS activity/elicitor	2–50-fold higher activity	Eulgem et al. (1999)
Hv:LOXA_P1, P2, P3	*loxA*	transgenic barley leaves	GUS activity/methyl jasmonate (MeJA)	11.9–19.4-fold higher expression	Rouster et al. (1997)
Cr:CPR_P1	*crp*	transgenic tobacco plants	GUS activity/elicitor	Essential for controlled expression	Cardoso et al. (1997)
Nt:BGLUCANASE_P1, P2	*gln2, gglb50*	transgenic tobacco plants, transgenic tobacco plants (1 month-old)	GUS activity/tobacco mosaic virus, salicylic acid, ethephon (ethylene), water	2–10-fold higher activity	van de Rhee et al. (1993), Livne et al. (1997)

Nt:PR2D_P1, P2, P3	*pr2d*	transgenic tobacco plants	GUS activity/tobacco mosaic virus, salicylic acid, water	2–18-fold higher activity	Hennig et al. (1993)
Nt:PR2D_P2	*pr2b*	transgenic tobacco plants	GUS activity/tobacco mosaic virus, salicylic acid, water	five- to nine-fold higher activity	van de Rhee et al. (1993)
Np:GN1_P1	*gn1*	transgenic tobacco plants	GUS activity/SA, ethylene, water, elicitor, wounding	1.7–21-fold higher activity	Castresana et al. (1990)
At:CEL5_P1	*At1g22880*	transgenic *Arabidopsis* seedlings	GUS activity/auxin, ABA	Effective GUS staining activity	del Campillo et al. (2004)
As:PHYA3_P1, P2	*phyA3*	transgenic etiolated rice seedlings (2 day-old)	CAT activity/far-red light	five-fold higher activity	Bruce and Quail (1990)
St:GLUB_P1	*gluB8-1-3*	transgenic potato plants, transgenic tobacco plants	GUS activity/pathogen, tobacco mosaic virus, elicitor	2–12-fold higher activity	Mac et al. (2004)
Hv:GIII_P1	*GIII*	transgenic rice plants, transgenic rice calli	GUS activity/salicylic acid	Fragment length dependent GUS activity	Li et al. (2005)
Nt:PR1A_P1, P2	*pr1A*	transgenic tobacco plants	GUS activity/salicylic acid, tobacco mosaic virus	58–110-fold higher activity	Strompen et al. (1998), Uknes et al. (1993)
At:CYP85A1_P1	*cyp85A1*	transgenic *Arabidopsis* seedlings	GUS activity/Brassinolide	weak activity	Castle et al. (2005)
At:APX1_P1, P2, P3	*apx1*	transgenic *Arabidopsis* seedlings (2 week-old), (10 day-old)	GUS mRNA, GUS activity/ heat shock, methyl viologen, iron	Effective GUS staining	Storozhenko et al. (1998), Fourcroy et al (2004)
At:APX2_P1	*apx2*	transgenic *Arabidopsis* plants	LUC activity/hydrogen peroxide (H_2O_2), high light	ten-fold higher activity	Kimura et al. (2001)

(*continued*)

Table 5.1 (continued)

Promoter	*Gene*	Specificity	Reporter/inducer	Comment	References
At:APX2_P2	*apx1b*	transgenic tobacco mesophyll protoplasts	GUS activity/Heat Stress transcription Factor (HsfA2)	ten-fold higher activity	Schramm et al. (2006)
At:ATHB6_P1	*athb6*	transgenic *Arabidopsis* seedlings, transgenic *Arabidopsis* plants	GUS activity/drought, abscisic acid (ABA), salt	increased GUS staining with high level expression	Söderman et al. (1999)
At:CYP85A2_P1	*cyp85A2*	transgenic *Arabidopsis* seedlings	GUS activity/brassinolide	promoter activity down-regulated by brassinolide	Castle et al. (2005)
At:ELIP2_P1	*elip2*	transgenic *Arabidopsis* seedlings (10–14 day-old)	LUC activity/high light	100-fold increased expression	Kimura et al. (2001)
At:Fer1_P1	*fer1*	transgenic *Arabidopsis* plants, transgenic *Arabidopsis* cells	GUS activity/iron, senescence	17-fold derepression in response to 0.5 mM iron citrate	Tarantino et al. (2003)
At:Fer1_P2	*fer1*	transgenic *Arabidopsis* cells	GUS activity/iron	six-fold higher activity in response to 0.5 mM iron citrate	Petit et al. (2001)
Ca:Chi2_P1, P2, P3	*chi2*	transgenic tobacco	GUS activity/infection, mannitol, salt, NaCl, salicylic acid	1.5–4.5-fold higher activity	Hong and Hwang (2006)
Gm:Fer_P1, P2	*fer*	transgenic soybean leaves	LUC activity, GUS activity/ iron	iron dependent GUS activity	Wei and Theil (2000)
Hv:BLT101.1_P1, P2, P3	*blt101.1*	transgenic barley	GUS activity/low temperature	2.5-fold higher expression at low temperature	Brown et al. (2001)
Hv:BLT4.9_P1, P2, P3	*blt4.9*	transgenic barley	GUS activity/low temperature	2.5–6-fold higher expression at low temperature	Dunn et al. (1998)
POPLA:PAL1_P1	*pal1*	transgenic tobacco	GUS activity	Differential Gus activity	Gray-Mitsumune et al. (1999)

POPLA:PAL2_P1	*pal2*	transgenic tobacco, transgenic poplar	GUS activity	Differential Gus activity	Gray-Mitsumune et al. (1999)
Ta:GERMIN_P1	*gf-2.8*	transgenic tobacco seedlings, transgenic tobacco plants	GUS activity/heavy metal, cadmium, copper, cobalt, wounding, TMV	Gus activity detected after induction	Berna and Bernier (1999)
Zm:Fer1_P1	*fer1*	transgenic maize cells (BMS, Black Mexican Sweet)	GUS activity/iron	2.5–8-fold increase in activity	Petit et al. (2001)
At:FPS2_P1	*fps2*	transgenic *Arabidopsis* plants, transgenic *Arabidopsis* protoplasts	GUS activity	Differential Gus activity	Cunillera et al. (2000)
At:PAL1_P1, P2	*pal1*	transgenic *Arabidopsis* plants (3 week-old), transgenic tobacco plants (6 leaf stage)	GUS activity, GUS mRNA, PAL mRNA/ wounding, HgCl2, white light, elicitor, H2O2, mitomycin C (MMC)	30% increase in GUS activity	Ohl et al. (1990)
At:PDF1.2_P1	*pdf1.2*	transgenic *Arabidopsis* seedlings (10 day-old), transgenic *Arabidopsis* plants (4 week-old), transgenic tobacco seedlings (2 week-old), (6 week-old)	GUS activity/jasmonic acid (JA), pathogen, elicitor, methyl jasmonic acid (MeJA), paraquat, methyl viologen, rose bengal, TMV, ethylene	9–25-fold higher activity	Manners et al. (1998)
At:RAD54_P1	*atrad54*	transgenic *Arabidopsis* seedlings (1 week-old)	GUS mRNA/gamma irradiation	GUS activity detected after gamma irradiation	Osakabe et al. (2006)
At:THI1_P1, P2	*thi1*	transgenic *Arabidopsis* plants (14 day-old)	GUS activity/white light, flooding, salt, sugar deprivation	two to six-fold higher activity	Ribeiro et al. (2005)

(*continued*)

Table 5.1 (continued)

Promoter	*Gene*	Specificity	Reporter/inducer	Comment	References
Gm:SCAM4_P1	*cam-4*	transgenic *Arabidopsis* seedlings (2–4 day-old), transgenic *Arabidopsis* leaf protoplasts, transgenic *Arabidopsis* plants (4 week-old)	GUS mRNA, GUS activity/ salt, glycol chitin, Ca2+-ionophore A23187, elicitor, pathogen	3–15-fold higher activity	Park et al. (2004)
Le:LAT52_P1	*lat52*	transgenic tobacco mature pollen	GUS activity, LUC activity	Essential for full promoter activity	Bate and Twell (1998)
Zm:GAPC4_P1	*gapc4, gpc4*	transgenic tobacco leaves	GUS activity/anaerobic conditions	seven-fold higher activity	Geffers et al. (2001)
Nt:SAR8.2B_P1, P2, P3	*sar8.2b*	transgenic *Arabidopsis* plants (3 week-old)	GUS activity/Salicylic acid	4–31-fold higher GUS activity	Song et al. (2002)
Nt:PMT1A_P1	*pmt1a*	transgenic tobacco suspension cells	GUS activity/ (MeJA), ethephon	10–15-fold higher activity	Xu and Timko (2004)
Nt:G10_P1	*g10, tobacco late pollen gene g10*	transgenic tobacco pollen and leaves	GUS activity	Essential for full promoter activity	Rogers et al. (2001)
Ps:DRR206D_P1	*drr206-d, pi206*	transgenic tobacco plants (6-leaf stage)	GUS activity/Mitomycin C, actinomycin D, etoposide,H_2O_2, elicitor	Promoter only induced by natural tobacco Pathogen	Choi et al. (2001)
Nt:AP24_P1, P2	*ap24*	transgenic tobacco seedlings, transgenic tobacco plants	GUS activity/ethylene, NaCl, absicisic acid	2.5–13-fold higher Activity	Raghothama et al (1997)
At:OPR1_P1	*opr1*	transgenic *Arabidopsis* seedlings (2-week old)	GUS activity/Methyl jasmonate (MeJa), senescence	2–3.5-fold higher activity	He and Gan (2001)
Sc:OSML13_P1 Sc:OSML81_P1	*osml13 osml81*	transgenic potato plants	GUS activity/ABA, NaCl, SA, wounding, fungal infection, cold	Differential GUS activity	Zhu et al. (1995)
Sc:CI21A_P1, P2	*ci21A*	transgenic potato plants	GUS activity/low temperature (cold)	1.7–7-fold higher activity	Schneider et al. (1997)

There are a few limitations in the use of virus-derived promoters: first, the potential risk to human health from the genes of infective plant viruses (Hodgson 2000), and second, the ability of plant cells to recognize the inserted sequences of nonplant origin and inactivate them via "transcriptional gene silencing." Silencing is less common in promoters of plant origins.

Strong constitutive promoters of plant origin have also been isolated and used for the development of transgenic plants. Actin, a fundamental cytoskeleton component of the cell, is expressed in almost all the cells of a plant. The Act2 promoter, developed from *Arabidopsis* showed strong expression in all other parts except the seed coat, hypocotyl, ovary, and pollen sac (An et al. 1996). Similarly, rice Act1 promoter has also been developed (Zhang et al. 1991). Ubiquitins, a highly conserved protein family, are linked to many important cellular functions like chromatin structure and DNA repair. Maize ubiquitin 1 promoter (pUbi) has been successfully used for plant transformation of monocots (Weeks et al. 1993; Gupta et al. 2001) and has shown high levels of expression in actively dividing cells. Transgenic plants developed using Ubi.U4 promoter from *Nicotiana sylvestris* showed a three-fold higher activity when compared to the CaMV-based promoters. The ubiquitin-derived promoters perform very well in metabolically and mitotically active cells.

The constitutive action of a promoter has many drawbacks. Expression (or overexpression) of the transgene at a place where it is not expressed or expressed at a wrong time can have severe consequences on the growth and development of the plant. It can lead to enhanced susceptibility to some pathogens (Berrocal-Lobo et al. 2002) or decreased growth (Bowling et al. 1997). Another concern is the development of resistance by target insects against overexpressed toxins like, e.g., *Bt* toxin (Huang et al. 1999). For these reasons, it is ideal to strategically develop promoters that are "switched on" precisely when they are needed.

5.4.1.2 Nonconstitutive (Tissue-Enhanced) Promoters

Development of a new trait or value-addition of a previously existing one by genetic engineering requires the development of transgenes that are under control and expressed in a tissue-specific, developmental, or inducible manner. This will conserve energy and circumvent the drawbacks associated with constitutive expression to a large extent. It is more realistic to call these promoters tissue enhanced rather than tissue specific as their expression may not be confined to a specific tissue or plant part. Tissue-enhanced gene expression pattern is achieved as a result of several factors executed at various levels of gene control. Also, the more distant 5′-*cis* acting enhancer element may be eliminated during isolation of the promoter, and there may not be effective functional interaction between the promoter *cis*-elements with the heterologous *trans*-acting factors present in the transgenic host plant. Thus, the development of such promoters can be very complex and difficult. Because of these complexities, it is preferable to use promoters from homologous or closely related plant taxa, and knowledge about

the functionality of both homologous and heterologous promoters in target crop plants is essential.

Roots

These promoters are of high interest as they can be used for multiple applications. Expression of proteins responsible for resistance against drought and salt tolerance, resistance to bacterial (or fungal) pathogens or nematodes (Atkinson et al. 2003), and phytoremediation (Grichko et al. 2000) can improve crop yield. Although root-specific promoters have been isolated in plants (Yamamoto et al. 1991; Liu and Ekramoddoullah 2003), other stress-related studies have identified many candidate genes and their promoters. In maize, bacterial promoters are more popular (Qing et al. 2009). *Agrobacterium rhizogenes* causes hairy root disease in dicots. The promoters for rooting loci genes (*rol*) present in the root-inducing (Ri) plasmids are largely studied because of their root-mediated transformation and expression of the transgenes. Most important of the rol promoters is the rolD promoter, which has been much utilized (Stearns et al. 2005; Jayaraj et al. 2008) and extensively used in nitrogen assimilation studies (Fraisier et al. 2000; Fei et al. 2003). Very high levels of rolD promoter activity have been reported earlier (Elmayan and Tepfer 1995), but recently, in a comparative study, it was reported that *Arabidopsis* ubiquitin promoter (UBQ3) has the highest expression in roots (Wally et al. 2008). The domain A (−90 bp upstream) of CaMV 35S also shows root-specific activity (Benfey and Chua 1989; Benfey et al. 1990).

TobRB7, a putative membrane channel aquaporin, is another valuable plant based root-specific promoter isolated from tobacco (Yamamoto et al. 1991). Root-specific activity of this promoter was observed within 2 days of germination. Recently, a novel gene has been isolated from tomato, having very high expression levels in roots (*SlREO*); the 2.4-kb region representing the *SlREO* promoter sequence showed strict root specificity (Jones et al. 2008).

Root Nodules

Root nodules are formed as a result of an endosymbiotic association between *Rhizobium* and other species of bacteria with leguminous host plants. Within the nodule, the bacteroids fix atmospheric nitrogen that is used by the plant and in return, receive carbon substrates from the plant. This type of symbiosis is well studied. Leghemoglobin is an oxygen-binding protein synthesized in the nodule. The expression of leghemoglobin coincides with the nitrogen fixation in the nodule. Thus, this promoter can be used in nodules to increase nitrogen assimilation. The leghemoglobin promoter *glb3* from *Sesbania rostrata* was expressed in *Lotus corniculatus* and tobacco-harboring chimeric *glb3-uidA* (gus) gene fusions (Szabados et al. 1990; Szczyglowski et al. 1996). A 1.9-kb fragment of the *glb3* 5′-upstream

region was found to direct high level of nodule-specific β-glucuronidase (GUS) activity in *L. corniculatus* that is restricted to the *Rhizobium*-infected cells of the nodules. In tobacco (a nonleguminous plant), the activity was restricted primarily to the roots and to phloem cells of the stem and petiole vascular system. A deletion analysis revealed that the region between −429 and −48 bp relative to the ATG was effective for nodule-specific expression.

Tubers

Tubers are storage organs in roots and are staple food source in many countries of the world. Improvement of tuber nutrition value, resistance toward infectious disease and pesticides can be manifested by using tuber-specific expression of transgenes. Patatins are glycoproteins that are one of the major products found in potato tuber. These are tuber- specific and can be induced by sucrose (Jefferson et al. 1990). The patatin promoters *Pat1* and *Pat2* were used to overexpress transgenes from the minipathway, of bacterial origin, to drive the synthesis of β-carotene (Provitamin A) in vitamin-A-deficient tubers of potato (Diretto et al. 2007). To enhance the metabolism of the environmental contaminants in tubers the rat P450 monooxygenase gene (*CYP1A1*) was overexpressed in tubers of potato, also under the control of patatin promoter (Yamada et al. 2002). High transgene expression was seen in developing tubers, and the amount of residual herbicides was much lower than that in nontransgenic plants, indicating that the transgenic plant metabolized and detoxified the herbicides. The processing quality of potato products (fries and chips) was increased by overexpressing transgenes in potato tuber under the control of TSSR (tuber-specific and sucrose-responsive) sequence from potato class I patatin promoter (Zhu et al. 2008). It was also demonstrated that tuber-specific expression of the native and slightly modified *MYB* transcription factor gene *StMtf1*(M) activates the phenylpropanoid biosynthetic pathway. The transgenic potato tubers contained four-fold increased levels of caffeoylquinates, including chlorogenic acid while also accumulating various flavonols and anthocyanins (Rommens et al. 2008). An 800-bp 5′ upstream sequence of the granule-bound starch synthase (*GBSS*) gene from potato was highly expressed in stolons and tubers (Visser et al. 1991), where the activities of the transgene in these two organs were 3–25-fold higher than the expression of the CaMV-*GUS* gene. The *GBSS* gene promoter was also used to obtain tuber-specific high expression of *AmA1*, a nonallergenic seed albumin gene from *Amaranthus hypochondriacus* in potato (Chakraborty et al. 2000). Two promoters, sporamin and β-amylase, have been well characterized in sweet potato (Maeo et al. 2001). The sweet potato sporamin promoter was found to control the expression of the *E. coli appA* gene in transgenic potato, which encoded a bifunctional enzyme exhibiting both acid phosphatase and phytase activities (Hong et al. 2008). Phytase expression levels in transgenic potato tubers were stable over several cycles of propagation. The study demonstrated that the sporamin promoter can effectively direct high-level recombinant protein expression in potato tubers. Moreover,

overexpression of phytase in transgenic potato offers an ideal feed additive for improving phytate-Phosphorous digestibility in monogastric animals along with improvement of tuber yield, enhanced Phosphorous acquisition from organic fertilizers, and has a potential for phytoremediation.

Leaves

The light received in the environment can be roughly categorized as UV, visible, and far red. Three classes of photoreceptors have been identified in higher plants: red light and far-red light absorbing phytochromes (PHYs), blue-light receptors, and UV-light receptors. In *Arabidopsis*, five members compose the PHY family of photoreceptors (PHY A-E) and at least three different blue light photoreceptors have been identified [cryptochromes (CRYs), NPH1, and NPL1] (Martínez-Hernández et al. 2002). These photoreceptors, with association of other molecular systems (transcription factors), control the expression of many genes at the transcriptional and post-transcriptional level. Two important transcription factors are basic Leucine zipper factor HY5 (Oyama et al. 1997) and bHLH factor PIF3 (Martínez-García et al. 2000).

The photosynthesis-associated nuclear genes (*PhANGS*), like the chlorophyll a/b-binding proteins (Cab) and the small subunit of Rubisco (RbcS), contain a number of *cis*-acting elements, the transcription of which is controlled by light. Some of the motifs like G, I, and GTI boxes are found in the promoter regions of many light-regulated genes (Giuliano et al. 1988; Green et al. 1988; Menkens et al. 1995). The LS5-LS7 region from the *Lemna gibba Cab19* gene (Kehoe et al. 1994) and the CGF-1 factor-binding site from the *Arabidopsis CAB2* gene (Anderson and Kay 1995) contain the GATA and GT-1 sequences; still these two regions are unable to activate transcription, thus suggesting that additional regulatory elements are involved. This has led to the general hypothesis that light-responsive elements (LREs) are formed by the aggregation of different transcription factors. It has also been shown that artificial sequences composed of paired combinations of tetrameric repeats of G- and GATA boxes or GT1- and GATA-boxes, but not multimers of a single motif, function as LREs (Puente et al. 1996). Monocot rbcS promoters have different *cis*-acting elements and have different patterns of spatial expression than dicots. The C3 rbcS is specifically expressed in mesophyll cells, while the C4 rbcS is expressed in bundle sheath cells, and not in mesophyll cells (Nomura et al. 2000; Patel and Berry 2008). Overexpression of *Arabidopsis* phytocrome A (PHYA), under the control of rbcS promoter, in commercially important rice varieties produced an increased number of panicles per plant (Garg et al. 2006). In an attempt to obtain high-level production of intact *Acidothermus cellulolyticus* endoglucanase (E1) in transgenic tobacco plants using the constitutive (Mac) as well as light-inducible tomato Rubisco small subunit promoter (RbcS-*3C*), it was observed that RbcS-3 promoter was more favorable for E1 expression in transgenic plants than the Mac promoter (Dai et al. 2005). Moreover, by replacing *RbcS*-3C UTL with AMV RNA4 UTL, E1 production was enhanced more than two-fold. In a

comparative study of the expression pattern of heterologous RbcS, RbcS3CP (0.8 kbp) from tomato, SRS1P (1.5 kbp) from soybean, and CaMV 35S in apple, it was found that the activity of SRS1P promoter was strictly dependent on light, whereas that of the RbcS-3C promoter appeared not to be so (Gittins et al. 2000). Later rolCP and CoYMVP were used for expression in vegetative tissues of apple; the CoYMV promoter was slightly more active than the rolC promoter, although expression was at a lower level than the CaMV 35S promoter (Gittins et al. 2003). The results indicated that both promoters could be suitable to drive the expression of transgenes to combat pests and diseases of apple that are dependent on interaction with the phloem.

The Cab proteins are highly expressed in green tissues and are often associated with other proteins to form the light-harvesting complex (Lhc). The expression pattern of the *Cab* gene in plants is different from that of the RbcS under certain physiological conditions as response to light quality and diurnal rhythm is different between these two genes (Ha and An 1988). Upon analysis of regulatory elements of *Cab-E* gene from *Nicotiana plumbaginifolia*, three positive and one negative *cis*-acting elements that influence photoregulation were found and of the three positive promoters two (PRE1 and PRE2) confer maximum level of photoregulation (Castresana et al. 1988). Tobacco plants when transformed with a chimeric gene encoding the A1 subunit of cholera toxin regulated by wheat Cab-1 promoter greatly reduced susceptibility to the bacterial pathogen *Pseudomonas tabaci* (Beffa et al. 1995).

Both RbcS and Cab are members of a multigene family and are expressed at very high level in green tissues (especially leaves), but many genes within the family contribute to the total protein content. Thus, the level of transgene expression is potentially dependent on the gene promoter used, so a strong green tissue-specific promoter from a single gene family will be most valuable.

Flowers

A substantial economic market has developed for cut flowers. Floral-specific promoters are therefore important for use in engineering transgenic flower varieties that may enhance vase life, visually appealing character of the flowers (reviewed by Mol et al. 1999) along with fragrance of interest and resistance to pests (Dolgov et al. 1995). The UEP1 promoter from Chrysanthemum when fused with a reporter gene (*GUS*) and transformed back into Chrysanthemum showed very high levels of expression in ray florets and three-fold lower expression in disk florets (Annadana et al. 2002). The activity of UEP1 promoter in ray florets is limited to petal tissues and does not extend into the tube of the petal or the sexual whorls of the floret. The promoter had 50-fold higher expression when compared with double CaMV-based promoters in petal tissues of ray florets (Annadana et al. 2002). This study also showed that CER6 promoter, associated with the wax biosynthesis pathway, had very high expression in ray florets, but the expression was much variable when compared to the UEP1 promoter.

Flavonoids are common color pigments in flowers and also perform many other functions including signaling and UV-protection. Engineering of the flavonoid biosynthetic pathway has led to the development of blue carnations (Holton 1995) and blue roses (Katsumoto et al. 2007). Chalcone synthase (CHS) genes as well as promoters have been studied extensively. The French bean CHS15 promoter showed expression in flowers and root tips of transformed tobacco plants (Faktor et al. 1996). In flowers, expression was confined to the pigmented part of petals and was induced in a transient fashion. Floral and root-specific expression required two conserved motifs, G-box and H-box, located near the TATA box. To evaluate the tissue-specific role of these motifs, a 39-bp DNA fragment containing the two motifs was prepared and fused with minimal promoters of CHS15 and CaMV 35S along with a marker gene (*GUS*). Tobacco plants were transformed and it was observed that the 39-bp polymer confers, upon both minimal promoters, a high level of expression that follows the typical tissue-specific expression pattern (Faktor et al. 1997). A chromoplast-specific carotenoid-associated gene (*OgCHRC*) and its promoter (Pchrc) was isolated from an orchid species (*Oncidium*), which showed very high and had flower-specific expression (Chiou et al. 2008).

Pistils

Pistil comprises the female part of the flower and includes stigma, style, and ovary. Identification of ovule-specific promoters is useful for the genetic engineering of crops with a variety of desirable traits, such as genetically engineered parthenocarpy, female sterility, or seedless fruits. The *SK2* gene from *Solanum tuberosum* encodes a pistil-specific endochitinase; the promoter from this gene was fused with a reporter (*GUS*) and when transformed back into potato, high-level expression specific to pistil was observed (Ficker et al. 1997). The 2.4-kb 5′-flanking region of the pistil-specific thaumatin gene (*PsTL1*) from Japanese pear, when transformed in tobacco, showed high expression in pistil, low in anther, and no detectable expression in the floral organs or the leaves. The promoter for *Arabidopsis AGL11* gene, when transformed back into *Arabidopsis,* showed high expression in the center of the young ovary, while expression was not seen in vegetative plant tissues, sepals, petals, or androecium (Nain et al. 2008).

Pollen/Anther

Anther as well as pollen-specific expression can be classified into “early” and “late” phases. The “early” phase comprises genes that are expressed during anther development and sporophytic tissue formation, while “late” phase involves expression during gametophyte generation and pollen formation/maturation. The 122-bp 5′ region of a tapetum-specific gene (*TA29*) isolated from tobacco programmed

tapetum-specific expression as seen by fusing this promoter with a reporter (Koltunow et al. 1990). The expression increased in the developing anther and decreased as the microspores began to mature into pollen. The *TA29* promoter, fused with RNase (*barnase*), has been used to develop nuclear male-sterile plants (Mariani et al. 1990). In a comparative analysis, expression patterns of Bp4 promoter from rapeseed and the NTM19 promoter from tobacco were studied in transgenic tobacco (Custers et al. 1997). The Bp4 promoter became active only after the first pollen mitosis and not in the microspores, while the NTM19 promoter turned out to be highly microspore specific and directed very high level of GUS expression to the unicellular microspores; more importantly both the promoters were expressed only in the male germline (Custers et al. 1997). In *indica* rice, promoter of OSIPA was active during the late stages of pollen development and remained active till anthesis, whereas OSIPK promoter was active at a low level in developing anther till the pollen matured. OSIPK promoter activity diminished before anthesis. Both the promoters showed a potential to target expression of the genes of interest in developmental stage-specific manner and could help engineer pollen-specific traits in transgenic crops (Gupta et al. 2007). The anther- and tapetum-specific gene *TomA108* was present in as single copy per haploid genome of tomato. The fusion of β-glucuronidase to the TomA108 promoter demonstrated that the promoter was highly active from early meiosis to free microspores production in tapetum of tobacco (Xu et al. 2006).

Recently, a gene from pea, *PsEND1*, showed very high and early expression in anther primordium cells. Later *PsEND1* expression became restricted to the epidermis, connective, endothecium, and middle layer, but it was never observed in tapetal cells or microsporocytes. On fusion of the PsEND1 promoter region to the cytotoxic *barnase* gene to induce specific ablation of the cell layers, where the PsEND1 was expressed it produced male-sterile plants in tobacco and tomato (Roque et al. 2007). The PsEND1-*barnase* gene is quite different from other chimeric genes previously used to obtain male-sterile plants. The tapetum-specific promoter produces the ablation of specific cell lines during the initial steps of the anther development, but this chimeric construct (PsEND1-*barnase*) arrests the microsporogenesis before differentiation of the microspore mother cells and so, no viable pollen grains are produced. This strategy represents an excellent alternative to generate genetically engineered male-sterile plants. The PsEND1 promoter has high potential to prevent undesirable horizontal gene flow in many plant species (Roque et al. 2007). Two anther-specific cDNAs (designated *GhACS1* and *GhACS2*) encoding acyl-CoA synthetases (ACSs) isolated from cotton flower cDNA library were seen to accumulate in developing anthers. *GUS* expression controlled under the GhACS1 promoter showed high and specific expression in primary sporogenous cells, pollen mother cells, microspores, and tapetal cells (Wang and Li 2009).

Compared to "early" phase genes a few "late" phase genes have also been characterized. The promoter of tomato *Lat52* gene showed pollen-specific activity

when transformed to tomato, tobacco, and *Arabidopsis* plants. Its expression was also correlated with the onset of microspore mitosis and increased progressively until anthesis (Twell et al. 1990). The elements necessary for expression in transgenics were present within 600 bp of the 5′ flanking region. The promoter sequence of *BAN215-6* gene from Chinese cabbage (*Brassica campestris*) showed high similarity with the *Lat52* gene (Kim et al. 1997). Expression studies, by *Agrobacterium*-mediated transformation of tobacco plants, revealed that 383 bp of the *BAN215-6* promoter region was sufficient for the anther-specific expression. The expression level was increased during anther development, reaching highest levels in mature pollens (Kim et al. 1997). The promoter of a maize pectin methytransferase gene (*ZmC5*) was found to be expressed specifically in late pollen development when transformed to tobacco plants (Wakeley et al. 1998). By genome walking PCR, a novel β-mannase gene (*LeMAN5*) was discovered in tomato, which is involved in cell wall disassembly and degrading mannan polymers. The 5′-upstream region of this endo-β-mannanase gene contained four copies of the pollen-specific *cis*-acting elements POLLEN1LELAT52 (*AGAAA*). The expression of the putative LeMAN5 promoter region (−543 to +38) in transgenic *Arabidopsis* was detected in mature pollen, sporangia, discharged pollen, and elongating pollen tubes (Filichkin et al. 2004).

Fruit

Fruits are one of the best delivery vehicles for value-added nutrients and other characters like increasing shelf-life, development of oral vaccines, etc. and there has always been a need for fruit-enhanced gene expression. The promoters of fruit-specific genes, especially fruit ripening genes, have been sought after. The ACC (1-aminocyclopropane-1-carboxylate) oxidase gene, the *E8* gene, and polygalacturonase (PG) genes are all fruit-ripening-specific promoters and have been characterized from apple and tomato (Montgomery et al. 1993a,b; Nicholass et al. 1995; Atkinson et al. 1998). The ACC oxidase gene is induced by application of ethylene, and fragments of 1,966 and 1,159 bp of the 5′ region showed both fruit and ripening specificity, whereas for the *PG* gene promoter, fragments of 1,460 and 532 bp conferred ripening-specific expression in transgenic tomato fruit (Atkinson et al. 1998). The promoter of the *E8* gene of tomato is by far the most important fruit-ripening-specific promoter. It has been successfully applied in a number of instances including enhancement of aroma of tomato by expressing *Clarkia breweri* S-linalool synthase gene (*LIS*) (Lewinsohn et al. 2001), fruit-specific expression of viral proteins (Sandhu et al. 2000), and cholera toxin gene (*CTB*) in an effort to make edible vaccines (He et al. 2008). The tomato *PG* gene is also associated with fruit ripening and its promoter was successfully employed to overexpress a bacterial phytoene synthase gene resulting in increased carotenoid content (Fraser et al. 2002) and a lemon basil α-zingiberene synthase gene (*ZIS*) in tomato fruit to increase both mono- and sesqui-terpene contents (Davidovich-Rikanati et al. 2008).

Seeds

Like fruits, seeds are also an excellent vehicle to pack transgenic products. Seed-specific transgenic technology can be used to enhance nutrient quality, production of pharmaceutical compounds, edible vaccines, etc. The genes expressed at very high level in the seeds are seed storage proteins and these have become the target of choice. Promoters for dicots as well as monocots have been extensively studied and several seed-specific elements have been characterized. The promoter region of soybean β-conglycinin was expressed in the embryo during the mid to late stages of seed development (Chen et al. 1989). The 2.4-kb upstream region of the sunflower *Helianthinin* gene (*HaG3-A*) also conferred high embryo-specific expression in transgenic *Arabidopsis* (Nunberg et al. 1994). The 0.8-kb fragment of the 5′ β-phaseolin gene of French bean (*Phaseolus vulgaris*) showed strong, temporally regulated, and embryo-specific expression in transgenic tobacco plants (Bustos et al. 1989). The expression pattern of the promoter fragment (1,108 bp) of the α-globulin gene in cotton was studied in transgenic cotton, *Arabidopsis*, and tobacco. Expression was initiated during the torpedo stage of seed development in tobacco, *Arabidopsis*, and during cotyledon expansion stage in cotton. The activity increased sharply until embryo maturation in all the three species. Expression was not detected in stem, leaf, root, pollen, or floral bud of transgenic cotton, thus confirming the high seed specificity of the promoter (Sunilkumar et al. 2002).

For monocots, several seed-specific promoters have been used successfully to incorporate many traits. The promoter region of the endosperm-specific protein hordein (D and B hordein) from barley has been well characterized in transgenic rice, barley, and wheat (Furtado et al. 2008, 2009). Six promoters (GluA-1, GluA-2, GluA-3, GluB-3, GluB-5, GluC) of seed storage glutenin genes were isolated from rice and their expression potential was checked in transgenic rice. The GluA-1, GluA-2, and GluA-3 promoters directed expression in the outer portion of the endosperm, while GluB-5 and GluC promoters directed expression in the whole endosperm. The GluB-3 promoter directed expression solely in aleurone and sub-aleurone layers, while maximum activity was pertained to the GluC promoter (Qu et al. 2008). Recently, edible vaccines are being made in transgenic rice against house dust mite allergy (Yang et al. 2008) and Japanese cedar pollen allergen (Yang et al. 2007) under the control of GluB-1 promoter and cholera toxin B subunit under the control of wheat Bx17 promoter containing an intron of the rice *act1* (Oszvald et al. 2008). The zein promoters from maize have been used for many applications. Transgenic maize with enhanced provitamin A content in the kernel was developed by endosperm-specific expression of the bacterial genes (*crtB* and *crtI*) under the control of a "super γ-zein promoter" (Aluru et al. 2008). Increase of total carotenoids was up to 34-fold with a preferential accumulation of β-carotene in the maize endosperm. The *phyA2* from *Aspergillus niger* was successfully expressed in maize seeds using the maize embryo specific globulin-1 promoter. The transgenic seeds showed a 50-fold increase in phytase activity (Chen et al. 2008). The developed maize hybrids had improved phosphorus availability for pig and poultry feed.

5.5 Factors Affecting Stability and Level of Transgene Expression

5.5.1 SAR/MAR Effect on Transgene Expression

Transgenic plants often display the chromosomal position effect, which results because of transgene integration events taking place within euchromatin, producing irregular and mixed expression. The pre-existing chromatin structure at the site of integration ultimately determines the expression level, acting either as an enhancer or as a silencer (Taddei et al. 2004). The chromosomal position effect can be prevented if the transgene is flanked by matrix attachment regions (MARs) also known as scaffold attachment regions (SARs) which are DNA elements that bind to the nuclear matrix (Mirkovitch et al. 1984; Allen et al. 2000). The location of MARs within transcription regulatory elements suggests that MARs may serve to bring these DNA sequences in proximity to the scaffold, thereby promoting enhancer and promoter activity by facilitating interaction with transcription factors (Nardozza et al. 1996). This inference is supported by loop domain model studies in which different expression profiles were observed on comparative analysis of transgenes that were flanked by MARs and those lacking it. Transgenes lacking MARs are influenced much by the surrounding chromatin structure; their expression levels are also dependent on local chromatin state. Transgenes that are flanked by MARs act independent of local chromatin state; thus, multiple copies of MAR-flanked transgene insertion might proportionally increase expression (Gasser and Laemmli 1986; Stief et al. 1989).

During gene activation chromatin structure becomes relaxed and the DNA is more accessible to DNase I. MARs that flank the chromatin loop domain function as the boundaries to differentiate active from inactive chromatin (Martienssen 2003). Later, on the basis of this inference, comparative study of the higher order chromatin structure and their accessibility to DNase I was performed in *Arabidopsis* and maize nuclei resulting in 45-Kb and 25-Kb domains, respectively, (Paul and Ferl 1998). It was reported that transgenic plants containing the synthetic MAR (sMAR) sequences derived from the MAR 3′ end of the immunoglobulin heavy chain (IgH) enhancer, exhibited high levels of expression compared to transgenic plants that lacked the sMARs (Nowak et al. 2001). A diversity of promoters and MAR sequences has been used to analyze transgene expression. Mankin et al. (2003) analyzed the effects of a MAR, from the tobacco *RB7* gene on transgene expression from six different promoters in stably transformed tobacco cell cultures. The presence of MARs flanking the transgene increased expression of constructs based on the constitutive CaMV 35S, NOS (nopaline synthase 5′ region), and OCS (octopine synthase 5′ region) promoters (Mankin et al. 2003). Expression from a heat-shock induced promoter also increased five- to nine-folds, and MARs did not cause expression in the absence of heat shock (Schöffl et al. 1993). The effect of MAR fragments from tobacco gene transformed to two hybrid poplar

clones and in tobacco plants was analyzed and found that MARs increased expression approximately ten- and two-fold, respectively, 1 month after cocultivation with *Agrobacterium*. Apart from gene expression, increased frequency of kanamycin resistance was also reported in poplar shoots (Han et al. 1997).

Different studies on MAR function in plant transgene expression provided interesting conclusions. The effect of MAR on transgene expression is analyzed only after the integration of transgene construct within plant genome. Enhancement of transgene expression has been reported in MARs containing stably transformed plant cell lines of soybean Gmhsp 17.6.L (Schöffl et al. 1993), yeast ARS1 (Allen et al. 1993; Vain et al. 1999), tobacco and rice Rb7 (Allen et al. 1996), tomato HSC80 (Chinn and Comai 1996), bean phaseolin (van der Geest et al. 1994), maize Adh1, Mha1 (Brouwer et al. 2002), and *Arabidopsis* ARS (Liu and Tabe 1998).

In a nutshell, MARs are not highly conserved but possess AT-rich DNA motifs of 100–3,000 bp containing binding sites for DNA topoisomerase II, DNA helicase, and DNA polymerase and thus are involved in structural organization of the genome. The loops created by MARs are topologically independent units of gene regulation and were found to facilitate the transcription of genes by changing topology along with less-condensed chromatin structure. The transgene constructs containing MARS are observed to create its own chromatin domain favorable for transcription; thus, MARS can reduce variability of transgene expression and increase level of expression.

5.5.2 *Effect of 5′ and 3′ UTR Regions*

The use of a specific promoter, with or without one or more enhancers, does not necessarily guarantee the desired level of gene expression in plants. In addition to the desired transcription levels, other factors such as improper splicing, polyadenylation, and nuclear export can affect accumulation of both mRNA and the protein of interest. Therefore, methods of increasing RNA stability and translational efficiency through mechanisms of post-transcriptional regulation are needed in the transgenic approach.

With regard to post-transcriptional regulation, it has been demonstrated that certain 5′ and 3′ untranslated regions (UTRs) of eukaryotic mRNAs play a major role in translational efficiency and RNA stability. For example, the 5′ and 3′ UTRs of tobacco mosaic virus (TMV) and alfalfa mosaic virus (AMV) coat protein mRNAs can enhance gene expression 5.4-fold and three-fold, respectively, in tobacco plants (Zeyenko et al. 1994). The 5′ and 3′ UTRs of the maize alcohol dehydrogenase-1 gene (*adh1*) are required for efficient translation in hypoxic protoplasts (Bailey-Serres and Dawe 1996; Hulzink et al. 2002).

Experiments with various 5′ UTR leader sequences demonstrate that various structural features of a 5′ UTR can be correlated with levels of translational efficiency. It was reported that 5′ UTR elements are required for the high-level expression of pollen *ACT1* gene in *Arabidopsis* (Vitale et al. 2003). During the

process of initiation of translation 40S ribosomal subunit enters at 5′ end of the mRNA and moves linearly until it reaches the first AUG codon, whereupon a 60S ribosomal subunit attaches and the first peptide bond is formed. Certain 5′ UTR contain AUG codons in mRNA, which interact with 40S ribosomal subunit resulting in a weak context in terms of initiation codon, thus decreasing the rate of translation (Kozak 1991; Lee et al. 2009; Luttermann and Meyers 2009). Additionally, the 5′ UTR nucleotide sequences flanking the AUG initiation site on the mRNA have an impact on translational efficiency. If the framework of the flanking 5′ UTR is not favorable, part of the 40S ribosomal subunit fails to recognize the translation start site such that the rate of polypeptide synthesis will be slowed down (Kozak 1991; Pain 1996). Secondary structures of 5′ UTRs (e.g., hairpin formation) also obstruct the movement of 40S ribosomal subunits during their scanning process and therefore negatively impact the efficiency of translation (Kozak 1986; Sonenberg and Pelletier 1988). The relative GC content of a 5′ UTR sequence was shown to be the stability indicator of the potential secondary structure, high GC content indicated instability (Kozak 1991), and long UTRs exhibit a large number of inhibitory secondary structures. The translational efficiency of any given 5′ UTR is highly dependent upon its particular structure and optimization of the leader sequence, which has been shown to increase gene expression as a direct result of improved translation initiation efficiency. Furthermore, significant increase in gene expression has been produced by addition of leader sequences from plant viruses or heat-shock genes (Datla et al. 1993).

In addition to 5′ UTR sequences, 3′ UTR sequences of mRNAs also influence in gene expression and known to control nuclear export, polyadenylation status, subcellular targeting, and rates of translation and degradation of mRNA from RNases. In particular, 3′ UTRs contain one or more inverted repeats that can fold into stem-loop structures, which act as a barrier to exoribonucleases, and interact with RNA-binding proteins known to promote RNA stability (Gutiérrez et al. 1999). However, certain elements found within 3′ UTR were reported to be RNA destabilizing, one such example occurring in plants is the DST element which can be found in small auxin up RNAs (SAURs) (Gil and Green 1996). A further destabilizing feature of some 3′ UTRs is the presence of AUUUA pentamers (Ohme-Takagi et al. 1993).

The 3′ UTRs were demonstrated to play a significant role in gene expression of several maize genes. Specifically, a 200-bp 3′ sequence is responsible for suppression of light induction of maize small m3 subunit of the ribulose-1, 5-biphosphate carboxylase gene (*rbc*/*m3*) in mesophyll cells (Viret et al. 1994). Monde et al. (2000) observed that the *pet D3′*-UTR stem loop secondary structure was not able to form RNA-protein complex, essential for translational activity and thus acted as weak terminator required for RNA maturation. One 3′ UTR frequently used in genetic engineering of plants is derived from nopaline synthase gene (3′ *nos*) (Wyatt et al. 1993).

In certain plant viruses, such as alfalfa mosaic virus (AMV) and tobacco mosaic virus (TMV), the highly structured 3′ UTRs are essential for replication and can be folded into either a linear array of stem-loop structures, which contain several high-

affinity coat protein binding sites or a tRNA-like site recognized by RNA-dependent RNA polymerases (Olsthoorn et al. 1999).

5.5.3 *Effect of Introns*

Introns are the intragenic regions that are not translated into proteins. These noncoding portions are present in pre-mRNA and further removed by splicing to yield mature RNA. Introns contain acceptor and donor sites at either end as well as a branch point site, which is required for proper splicing by the spliceosome. The number and the length of introns vary widely among species and among genes within the same species. Introns with alternative splicing may introduce greater variability in protein sequences translated from a single gene. Introns also enhance the level of transgene expression in plants (Callis et al. 1987).

Recent studies provided several examples of introns, whose impact on expression is larger than that of the promoter from the same gene. Many genes with fully functional promoter are not essentially expressed at all but require an intron for their expression. A study in *Arabidopsis* showed that *PRF2* intron is required for full expression of a PRF2 promoter and the β-glucoronidase (*GUS*) and also to convert PFR5:*GUS* fusion from a reproductive to vegetative pattern (Jeong et al. 2006). Introns can increase the expression level through their enhancer element, an alternative promoter activity, or it can be independent of their conventional enhancer elements, i.e., intron-mediated enhancement (IME). The second intron of *Arabidopsis* agamous gene (*AG*) is a well-characterized enhancer-containing intron that can function in both orientations to force the expression of a reporter from a minimal promoter. The *Arabidopsis AG, STK, FLC* introns and wheat *VRN-1* intron act as enhancers. All these introns are large in size providing sufficient room for controlling elements and allow the establishment of stable chromatin conformation required for appropriate expression (Rose 2008).

The studies conducted by Morello et al. (2002, 2006) revealed the role of intron as an alternative promoter in rice. Presences of introns in promoterless genes drive weak expression; these introns are considered to contain promoters that are responsible for expression. The first intron acts as the alternative promoter as observed in *Ostub16* and *OsCDPK2* in rice, *PpAct1* and *PpAct5* in *Physcomitrella patens* and sesame, and *FAD2* in *Arabidopsis* (Kim et al. 2006; Weise et al. 2006).

IME of gene expression in plants indicates that the insertion of one or more introns in a gene construct results in increased accumulation of mRNA and protein relative to similar fusions that lack introns (Mascarenhas et al. 1990). The deletion studies of different introns such as maize *Adh1, Sh1* first intron, rice *Ostub A1* first intron, *Arabidopsis TRP1* first intron and *PRF2* intron1 revealed that no specific sequences were absolutely required and no conserved motif was found between

enhancing introns (Rose 2008). Sequence analog studies showed enhancement can be restored by substituting the U/GC-rich region of intron with similar sequence analog from another part of intron (Rose 2002). The mutation studies in *Arabidopsis TRP1* intron1 and maize *Sh1* intron1 revealed that IME is destroyed by simultaneous elimination of branch-points and the 5′ splice site, further indicating that splicing machinery is required for IME (Rose 2002). Additionally, the positions of introns also influence IME on gene expression. The most prominent is the location of the intron within the gene, i.e., the introns present in 5′ UTR of the rice *rubi3* gene was shown to enhance expression (Lu et al. 2008). The other significant position of intron is near the starting of the gene (Rose 2004; Chung et al. 2006). Presently, there are several examples of introns (e.g., first intron of *OsTua2, OsTua3, OsTub4,* and *OsTub6*) that can greatly influence both the amount and the actual size of the expression, attributing different patterns of expression to the different intron isotypes, thus generating the intron-dependent spatial expression (IDSE) profile (Gianì

Table 5.2 Introns affecting transgene expression in different plants

Intron	Specificity	Remark	Reference
COX 5c-1	*Arabidopsis*	Increased GUS expression level	Curi et al. (2005)
COX 5c-2			
Ubi7	Potato	Ten-fold higher expression	Garbarino et al. (1995)
Adh1	Maize	40–100-fold increase in expression	Callis et al. (1987)
Act1	*Arabidopsis*	High level of reproductive tissue expression	Vitale et al. (2003)
RBCS2	*Chlamydomonas reinhardtii*	Stable high-level expression	Lumbreras et al. (1998)
reg A3	*Volvox carteri*	Required for *regA* expression	Stark et al. (2001)
regA5			
STK	*Arabidopsis*	Intron-mediated promoter expression in ovules and septum	Kooiker et al. (2005)
VRN-1	Wheat	Essential for promoter activity	Fu et al. (2005)
Ostub 16	Rice	Required for maximum promoter activity	Morello et al. (2002)
OsCDPK 2	Rice	Required for promoter activity	Morello et al. (2006)
PpAct 1	*Physcomitrella patens*	11–18-fold higher expression	Weise et al. (2006)
PpAct 5			
PpAct 7			
Sh1	Maize	10–1,000-fold enhanced expression	Maas et al. (1990, 1991)
Gap A1	Maize	Required for full promoter activity	Donath et al. (1995)
Actin 3rd intron	Maize	IME	Luehrsen and Walbot (1991)
Hsp81	Maize	IME	Sinibaldi and Mettler (1992)
Act 1	Rice	IME	McElroy et al. (1990)
tpi	Rice	Required for promoter activity	Xu et al. (1994)

et al. 2009). The specificity of introns acts as enhancer, and alternative promoter, or mediates enhancement on the basis of their numbers and position in a gene construct. Some of the introns with defined specificity have been summarized in Table 5.2.

5.5.4 Role of Transcription Factors

Transcription factors are sequence-specific DNA-binding proteins that interact with the promoter regions of the target genes and modulate the rate of initiation of mRNA synthesis by RNA polymerase II (Gantet and Memelink 2002). The role of transcription factors in transgene expression is studied by overexpression and antisense technology. For highly conserved transcription factors such as MADS-box or the Myb-like transcription factors, generation of antisense plant is difficult since the target requires an antisense RNA homology of over 50 bp, which is not preferred. Also, due to the presence of highly conserved regions, the specificity of antisense RNA is significantly reduced (Cannon et al. 1990). High-level expression of a transcription factor in a transgenic plant cell might favor the binding of the transcription factor to low affinity binding sites and result in activation of gene expression from noncognate promoters.

To study the effect of transcription factor on transgene, the steroid-binding domain of the glucocorticoid receptor is fused to a plant transcription factor. The absence of ligand represses nuclear localization and DNA-binding activities of transcription factor. After induction, repression is relieved and active protein can rapidly enter the nucleus and exert its transcription factor function. A glucocorticoid-responsive GAL4-VP16 fusion protein has been used to induce the activation of a luciferase reporter gene in transgenic *Arabidopsis* and tobacco plants, either by growing the plants on nutrient agar containing dexamethasone or by spraying the plants with the inducing compound (Aoyama and Chua 1997).

The *Arabidopsis* transparent testa glabra (*ttg*) mutant plants are not able to produce trichomes, anthocyanins, and seed coat pigment but generate excess root hairs. Production of trichomes and anthocyanins could be restored by overexpression of the maize transcription factor *R* in a constitutive and inducible manner (Lloyd et al. 1994). An interaction study carried out between transcription factor (*myb305*) and its promoter-binding site in *PAL2* in transgenic tobacco plant revealed that when leaves were inoculated with a PVX-construct expressing *Myb305* reporter gene, expression increased (Sablowski et al. 1995). Thus, the ectopic expression of *Myb305* in infected tissue incites the higher expression of *GUS* reporter gene in transgenic tobacco plant with nonmutant *PAL2* promoter element.

Synthetic transcription factors are an assembly of multiple zinc finger domains designed to achieve better regulation of gene expression. It is estimated that *Arabidopsis* contains 85 genes that encode zinc finger transcription factors (Riechmann and Ratcliffe 2000). Such synthetic zinc finger transcription factors (*TFsZF*) can be custom designed for binding to any DNA sequence (Segal and Barbas 2001).

Furthermore, the addition of herpes simplex virus VP16 activation domain to the polydactyl six-zinc finger protein 2C7 increased the expression more than 450-fold in transgenic plants (Liu et al. 1997). Later, Van Eenennaam et al. (2004) constructed five, three-finger zinc finger protein (ZFP) DNA-binding domains which tightly bound to 9-bp DNA sequences located on either the promoter or the coding region of the *Arabidopsis GMT* gene. When these ZFPs were fused to a maize *opaque-2* nuclear localization signal and the maize *C1* activation domain, four out of the five resulting ZFP-TFs were able to up-regulate the expression of the *GMT* gene in leaf protoplast transient assays. The seed-specific expression of these ZFP-TFs was reported to produce heritable increase in seed α-tocopherol level in subsequent generations of transgenic *Arabidopsis*.

The transcription factors, *R* and *C1,* interact to regulate anthocyanin biosynthesis in the maize kernel (Grotewold et al. 2000). In a recent study, it was reported that ectopic expression of a conifer *Abscisic Acid Insensitive 3* (*ABI3*) transcription factor induced high-level synthesis of recombinant human α-L-iduronidase gene in transgenic tobacco leaves (Kermode et al. 2007). Transgenic rice with *DREB 1s*/*CBF* or *OsDREB 1A*/*1B* transcription factor interact specifically with *DRE*/*CRT* or *OsDRE cis*-acting elements and control the expression of many stress-inducible genes (Ito et al. 2005). In continuation, Zhao et al. (2009) reported on the role of transcription factors on abiotic stress where the expression of yeast *YAP1* gene in transgenic *Arabidopsis* resulted in increased salt tolerance. The *YAP1* contains a basic leucine zipper domain similar to that of Jun (Moye-Rowley et al. 1989), which is a component of mammalian *AP-1* transcription factor complexes. Nuclear *YAP1* regulates the expression of up to 70 genes that are related to oxidative stress caused by high salinity (Zhao et al. 2009).

5.5.5 *Effect of DNA Acetylation and Methylation*

The interaction of histones with DNA plays an important role in chromatin remodeling and consequently the activation or repression of gene expression (Tian et al 2005). Intrinsic histone acetyltransferases (HATs) and histone deacetylases (HDs, HDAs, HDACs) drive acetylation and deacetylation, respectively, thus providing a mechanism for reversibly modulating chromatin structure and transcriptional regulation (Jenuwein and Allis 2001). Hyperacetylation relaxes chromatin structure and activates gene expression, whereas hypoacetylation induces chromatin compaction and gene repression. Histone acetylation and deacetylation are reversible and therefore play a significant role in transcriptional regulation associated with developmental programs and environmental conditions. These include day-length (Tian et al. 2003), flowering (He et al. 2003), osmotic and oxidative stress (Brunet et al 2004, De Nadal et al 2004), and cell aging (Imai et al 2000).

Acetylation neutralizes the lysine residues on the amino terminal tails of the histones, thereby neutralizing the positive charges of histone tails and decreasing

their affinity to bind DNA. HATs are often associated with proteins forming coactivator complexes, stabilizing the chromatin in an open conformation and transcriptionally active state. These complexes are targeted to promoters by specific transcription factors, allowing the RNApol II holoenzyme to access the promoter DNA sequence, which results in activation of transcription and increased gene expression. Histone deacetylases (HDAC) ameliorate the affinity of histones for DNA as deacetylation of histone tails result in stronger interaction between the basic histone tails and DNA. HDACs are often associated with other proteins that are associated with chromatin condensation and repression of transcription. These corepressor complexes promote heterochromatin formation, blocking access of RNApol II, thus resulting in repression of transcription.

Arabidopsis has 18 members of putative histone deacetylase family (Pandey et al. 2002). Among them *AtHDA6* is responsible for silencing transgenes (Murfett et al. 2001), whereas *AtHD1* is reported to be a global transcriptional regulator throughout the development of *Arabidopsis* (Tian et al. 2003). The analysis of microarray data revealed that gene activation is associated with increased levels of site-specific histone acetylation, whereas gene repression does not correlate with the changes in histone acetylation or histone methylation. Many of the HDACs found in plants are *Rpd3*, *HD2*, *SIR2*, and their homologs (Chen and Tian 2007).

DNA methylation is known to play a role in plant gene silencing (Ng and Bird 1999). Methyl CpG-binding protein (MeCP2) was reported to be involved in the recruitment of HDAC to methylated DNA through a corepressor complex, which results in gene silencing. Earlier studies showed that hemimethylation results in inhibition of transient gene expression, whereas nonmethylated gene expressed normally (Weber et al. 1990). In one of the studies, a mutation isolated via a transgene reactivation screen in *Arabidopsis*, mom1, was thought to act downstream of DNA methylation signals in controlling silencing because it did not confer obvious methylation changes (Amedeo et al. 2000). In recent studies, Shibuya et al. (2009) reported that the *pMADS3* gene in petunia, specifically expressed in the stamen and carpels of developing flower, showed ectopic expression after introduction of intron 2. This is known as ect-pMADS3 phenomenon and is due to transcriptional activation based on RNA-directed DNA methylation (RdDM) occurring in a particular CG in a putative *cis*-element in *pMADS3* intron 2. The CG methylation was maintained over generations, along with *pMADS3* ectopic expression, even in the absence of RNA triggers. Transcriptional or post-transcriptional gene silencing was expected; instead, upregulated gene expression was observed (Shibuya et al. 2009).

Recently, the new Amplicon-plus targeting technology (APTT) has been developed to overcome the problems of post-transcriptional gene silencing and lower accumulation of transgenic protein. This technology uses a novel combination of techniques, i.e., expression of a mutated PTGS suppressor, P1/HC-Pro, with PVX (potato virus X vector) amplicon encoding a highly-labile L1 protein of canine oral papillomavirus (COPV L1). Appreciable amount of protein accumulation was achieved by targeting the L1 to various cellular compartments, by

creating a fusion between the protein of interest and different targeting peptides. Additionally, a scalable "wound-and-agrospray" inoculation method has been developed that allows high-throughput *Agrobacterium* inoculation of *Nicotiana tabacum* to facilitate large-scale application of this technology (Azhakanandam et al. 2007).

5.6 Conclusions

Genetic transformation of crops has opened a new dimension to increase production that benefits both producers and consumers. Its effect can be best utilized in less developed or developing countries where crop yield is severely affected by biotic and abiotic stress. Also, value addition of existing nutrients along with production of novel nutraceuticals will help alleviate nutrition-related deficiencies in famine-stricken countries. Apart from enhancing food value in crop species, transgenic technology can be used to develop visual marker systems to monitor crops and carry out fine scale studies of agricultural crops. Despite the hostility against genetically modified crops in Eastern Europe, many countries in Asia and North America have accepted transgenic crops. In the present scenario, some of the factors responsible for the control of transgene expression at different levels have been summarized in Fig. 5.1. The primary challenge lies with the detailed understanding of the underlying mechanism involved in gene expression, and there is a pressing need to study gene expression, especially its regulation.

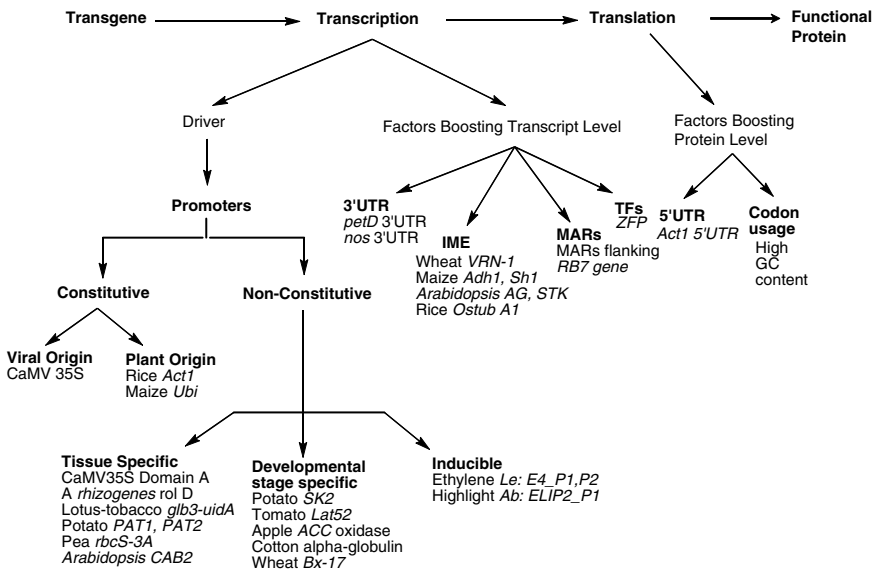

Fig. 5.1 Transgene expression and stability factors

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Chapter 6
Silencing as a Tool for Transgenic Crop Improvement

Pudota B Bhaskar and Jiming Jiang

6.1 Introduction

RNA silencing, also known as post-transcriptional gene silencing (PTGS) or RNA interference (RNAi), is a form of RNA degradation believed to be an important defense against foreign nucleic acids (Waterhouse et al. 2001). It was initially discovered in plants and was thought to function as part of a defense mechanism against viruses (Ratcliff et al. 1997). Subsequently, it was shown to be a common gene-silencing mechanism occurring in all eukaryotes, including plants and animals. The term RNAi was coined for the phenomenon when it was observed in the nematode *Caenorhabditis elegans* (Fire et al. 1998). However, this phenomenon of RNAi (PTGS) had actually been reported previously in transgenic petunia but was referred to as cosuppression, because transformation with a sense chalcone synthase transgene suppressed the expression of both the transgene and the endogenous gene (Napoli et al. 1990). It is now widely accepted that dsRNA is the effective trigger of PTGS/RNAi in plants and that this process operates by sequence-specific degradation (Kusaba 2004). Several milestones related to RNAi-based silencing are summarized in Table 6.1. In plants, cosuppression, PTGS, and virus-induced gene silencing (VIGS), all describe a homology-dependent gene-silencing phenomenon that involves what is more broadly known as RNAi. The science of RNAi broadly includes a few different and diverse RNA-silencing pathways that alter the expression levels of specific genes in plants, mediate the amplification and mobile signal mechanisms in RNAi pathways, and yield RNA-mediated DNA methylation (Baulcombe 2004; Lippman and Martienssen 2004).

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Table 6.1 Time-line showing the breakthroughs related to RNAi-based silencing technology

Year	Breakthrough	Publication
1990	Cosuppression of purple color in plants (*Petunia*)	Napoli et al. (1990)
1998	A concept of using double-stranded RNA for triggering silencing	Fire et al. (1998)
2000	First successful intron-based hairpin RNA construct for silencing	Smith et al. (2000)
2002	First successful attempt of using RNAi for crop improvement	Liu et al. (2002)
2005	RNAi shown to improve the quality aspects by organ-specific silencing	Davuluri et al. (2005)
2006	A concept of using RNAi for improving nematode resistance was demonstrated	Huang et al. (2006)
2007	First artificial microRNAs used for gene silencing for virus resistance	Qu et al. (2007)
2007	RNAi was demonstrated to be successfully applied for the insect resistance in crops	Baum et al. (2007), Mao et al. (2007)
2008	amiRNAs shown to trigger gene silencing in a crop plant, rice	Warthmann et al. (2008)

In this chapter, we will focus on the RNAi-mediated gene-silencing method available for the development of transgenic crop plants, with a focus on usable and deployable crop improvements. Readers interested in a more extensive deliberation on RNAi mechanism may consult a number of recent reviews (Waterhouse and Helliwell 2003; Vazquez 2006; Matzke et al. 2007; Ramachandran and Chen 2008; Eamens et al. 2008). Another gene-silencing-based method that has proven successful for crop improvement is tilling (Henikoff et al. 2004). However, desirable phenotypes obtained using this approach are not transgenic (a non-GM method) and hence not discussed in the current chapter.

6.2 Procedures for Development of RNAi-Based Transgenic Gene-Silencing Lines

RNA silencing is a homology-based process that is triggered by double-stranded RNA (*dsRNA*) and eventually leads to suppression of gene expression. Initially, sense or antisense RNA strands were used to mediate PTGS, most often with modest effects on gene expression (Waterhouse et al. 2001). Through cleavage by endonucleases called Dicers, dsRNAs are efficiently converted into small RNAs (~21–24 nt), which are then used to direct a sequence-specific degradation of cognate single-stranded RNAs (Vazquez 2006). Considerable research has been conducted to determine the most efficient silencing construct. Intron-containing hairpin RNA (hpRNA)-based vectors have been proven to be highly efficient for plant RNAi-based gene silencing (Smith et al. 2000). In a hpRNA vector, the target gene is cloned as an inverted repeat spaced with an intron and is driven by either a strong whole plant promoter, such as the 35S CaMV (dicots) or the maize

ubiquitin1 (monocots) or alternatively, an organ-specific silencing promoter. A spacer fragment between the arms of the inverted repeat is useful for increasing the stability of the vector in *Escherichia coli*, and using a splicable intron as a spacer has been shown to dramatically increase the frequency of strong silencing phenotypes (Smith et al. 2000). Typically, target-sequence inserts of 300–650 nt have been reported to provide reliably strong and frequent silencing in many crop plants (Helliwell et al. 2002; Matthew 2009).

Several types of RNAi-based vectors that make use of *Agrobacterium tumefaciens*-based plant transformation are available to the public and are being widely used. Predominantly, vectors developed by Waterhouse and colleagues at CSIRO, Australia, are supported by Gateway technology ™ and facilitate easy incorporation of target sequence in the sense and antisense direction with an intron between them (*http://www.pi.csiro.au/rnai*). Another set of RNAi vectors are available through the *Arabidopsis* Biological Resource center (ABRC, *http://www.arabidopsis.org*) and were donated by the Functional Genomics of Plant Chromatin Consortium (*http://www.chromdb.org*). Despite similar designs, these vectors differ in terms of selectable markers, type of promoters, and cloning strategies. Detailed information about the choices of different RNAi vectors is available (Matthew 2009; Preuss and Pikkard 2003).

RNAi is also affected by the transformation method. The most effective and heritable silencing has been achieved through stable transformation by *Agrobacterium or* particle bombardment (Waterhouse and Helliwell 2003). One drawback of this system is that if PTGS in the whole organism is desired, then stably transformed plants carrying these constructs must be generated. Nevertheless, stable transformation of RNAi constructs has currently been used as a tool for the genetic improvement in a variety of crops (Mansoor et al. 2006; Eamens et al. 2008). Currently, we have a much great understanding of the endogenous gene-silencing mechanism, providing knowledge that can be used to develop precisely targeted gene-silencing approaches.

6.3 Crop Improvements with Silencing Tools

6.3.1 RNAi for Resistance to Diseases and Pests

6.3.1.1 RNAi for Resistance to Viruses

Although the mechanism was not clear at that time, the effects of gene silencing in plants were first used in efforts to develop resistance to diseases, particularly those caused by viruses (Powell-Abel et al. 1986). This “pathogen-derived resistance” (PDR) was achieved by transforming plants with either genes or genetic fragments derived from the pathogen with the aim of blocking a specific step in the life or infection cycle of the pathogen. Most of the strategies used for PDR were shown to

be mediated by RNA, rather than protein, and led directly to the identification of PTGS – a phenomenon that is believed to be a form of antiviral defense (Voinnet 2001). An important finding recognized first in plants was that, once triggered, the silencing spreads throughout the organism by virtue of a gene-silencing signal, thus providing systemic rather than localized resistance (Voinnet et al. 1998). Unsurprisingly, virus-resistant transgenic plants are one of the first commercial applications resulting from gene-silencing technology.

The first demonstration that dsRNA mediates gene silencing in plants is the genetic study of Waterhouse et al. (1998). Transgenic plants were generated that expressed either the sense or antisense strand of a gene of potato virus Y (PVY). Both transgenic lines of tobacco were susceptible to PVY infection. However, progeny resulted from crosses between these susceptible tobacco lines showed resistance to PVY by generation of dsRNA. This suggests that two complementary RNAs transcribed from unlinked loci were able to anneal in the nucleus and induce a gene-specific suppressive state (Sharp 1999). This experiment first successfully demonstrated that dsRNA molecules are potent inducers of RNA silencing (Waterhouse et al. 1998).

Wang et al. (2000) first applied the deliberate use of RNA silencing for virus protection in the important cereal crop species barley. Barley yellow dwarf virus (BYDV) is a virus of global importance, as it infects and reduces yields of several crop species worldwide. An RNAi construct targeting the 5′ end of this virus was transformed into barley, and the lines obtained showed complete immunity to BYDV. The transgenic lines were field tested and have been commercially released. During the last several years, efforts to control various viruses infecting several crop plants have been reported. These include RNAi approaches to control single-stranded DNA viruses (Geminiviruses Pooggin et al. 2003) or RNA viruses (Poty viruses, Waterhouse et al. 1998). In some cases, a simultaneous silencing of diverse plant viruses was achieved by designing a single RNAi construct that targets multiple distinct viruses (Missiou et al. 2004). Viruses have been the obvious targets for RNAi technologies, as most viruses have single-stranded RNA genomes. Currently transgenic lines of several crop plants have been field tested or commercially released and continue to show very strong resistance to several plant viruses. For a complete, detailed list of the types of crops transformed for resistance to viruses and about the performance of virus-resistant transgenic crop plants, refer to a recent review by Fuchs and Gonsalves (2007).

6.3.1.2 RNAi for Resistance to Parasitic Nematodes

Plant-parasitic nematodes, in particular root-knot nematodes, are the most economically devastating group of plant-parasitic nematodes worldwide, attacking nearly all food and fiber crops grown. The inadequacy of current control methods provides an opportunity for transgenic approaches to make an important contribution to an integrated pest management strategy. One of the recent approaches to GM-mediated nematode resistance is host-induced RNAi gene silencing.

Plants can be engineered to produce dsRNAs that silence essential genes in the nematode. This dsRNA, or its siRNAs, would then be delivered from the plant to the nematode through ingestion of the plant cytoplasm. Once the siRNAs are inside the nematode, the RNAi process would inactivate the gene targeted by the dsRNA (Bakhetia et al. 2005).

This logic has been put into use for the first time in a recent report in which the goal was to engineer a host plant to become resistant to root-knot nematodes. Huang et al. (2006) targeted the parasitism gene, 16D10 from the nematode *Meliodogyne incognita* that encodes a small peptide necessary for the infection. This gene/peptide is secreted by the nematode into the plant roots and is thought to have an important role in the early signaling that occurs during feeding-site formation playing an important role in the plant-parasite infection. Expression of dsRNA directed against this gene (using a 35S promoter) resulted in *Arabidopsis thaliana* plants with a 70–90% reduction in the number of nematode eggs in the host plant. In other words, host plants showed resistance to multiplication of the nematodes. This range of resistance extended to four different types of root-knot nematodes. The range of resistance was unique and had not been previously obtained by any natural root-knot nematode resistance genes. This work is a good illustration of how fundamental RNAi mechanism might lead to engineering crop plants for nematode resistance. However, the method is still in the discovery phase involving only model plants, but this unique method might in the near future emerge as a viable and flexible means of developing novel and durable nematode-resistant crops for this devastating pathogen and others.

6.3.1.3 RNAi for Resistance to Insects

Transgenic expression of *Bacillus thuringenesis* (*Bt*) toxin in crop plants has proven to be a great success for pest control in several crops. However, many important pests are not susceptible to *Bt*-protection, and there is a danger that some crop pests might develop resistance to *Bt*. In integrated pest management, there is always a search for alternative and potentially complementary control strategies, particularly for agents that are more robust and/or broadly applicable (Gordon and Waterhouse 2007).

Recently, RNAi-mediated resistance has been exploited to control insect pests via the *in planta* expression of a dsRNA (Baum et al. 2007; Mao et al. 2007). This is in fact the first demonstration that the lessons learned from the use of RNAi in model organisms can be applied to real-life biological processes to obtain gains in controlling crop pests. The observations that ingested dsRNA can silence genes in both nematodes and *Drosophila* lead to the possibility of applying this technology to control crop insect pests. In this method, RNAi is induced in insects after ingestion of plant-expressed hairpin RNA.

This concept was demonstrated through managing a coleopteran insect pest. The Western corn rootworm (*Diabrotica virgifera*) is one of the most devastating pests in North America. The USDA estimates that the corn rootworm causes

US$1 billion in lost revenue each year, which equals $800 million in yield losses. Transgenic corn plants were engineered to express dsRNAs that target a western corn rootworm V-ATPase gene. V-ATPases are found in the plasma membrane of many organelles, such as endosomes, lysosomes, and vesicles, playing crucial roles in the function of these organelles. It was hypothesized that disruption of this enzyme is detrimental to the insects; thus, RNAi was directed to silence this gene. The transgenic plants expressing lethal insect dsRNAs were challenged with rootworm larvae and showed significant root protection compared with the nontransgenic control plants (Baum et al. 2007). No negative agronomic effects were seen in multiple generations of these transgenic plants.

Another successful RNAi strategy was reported by Mao et al. (2007) to improve resistance against a notorious lepidopteran insect pest, *Helicoverpa armigera,* commonly called cotton bollworm. First a cytochrome P450 gene was identified from cotton boll worm that acts as an antidote allowing the pest to resist the naturally occurring toxin, gossypol, produced by cotton plants. Transgenic tobacco and *Arabidopsis* plants were generated expressing dsRNAs against the bollworm cytochrome P450 gene. When larvae were fed leaves from these transgenic plants, the expression of the gene decreased and larval growth was retarded. It was recently reported that the engineered cotton plants showed partial resistance to cotton bollworm pest, as expected (Price and Gatehouse 2008).

This new method of in planta RNAi against feeding insects seems to have potential for future pest control strategies. A wide range of potential targets among various crop pests can be identified and targeted for suppression of gene expression to achieve increased resistance in plants.

6.3.2 RNAi to Enhance Quality Traits

6.3.2.1 Decaffeinated Coffee

The first example of using RNAi to improve the quality aspects of any crop plant was demonstrated in coffee. A cup of coffee, on average, contains 150 mg of caffeine, which can cause health problems for many people worldwide (Ogita et al. 2005). Consequently, decaffeinated coffee is preferred by buyers who are sensitive to caffeine and accounts for about 10% of the world coffee market (Ogita et al. 2005). The solvent extraction process used currently to chemically reduce the caffeine levels of coffee beans may leave undesired components in the decaffeinated beans. Therefore, coffee plants that produce caffeine-free beans have always been an objective. Currently, it takes ~20 years to develop a coffee variety with reduced levels of caffeine. Ogita et al. (2003) addressed this problem with an RNAi strategy. Coffee plants were transformed with RNAi constructs to silence the theobromine synthase gene in the caffeine biosynthetic pathway. The transgenic plants obtained showed a 70% reduction in caffeine content compared to

nontransformed coffee plants, and no phenotypic abnormalities were reported. While transgenic "decaf" lines have yet to be commercialized, this research has provided the first successful example of metabolic engineering of the alkaloids for quality improvement in crop plants.

6.3.2.2 Reduction of Toxic Gossypol in Cotton

One of the recent, dramatic applications of RNAi has been the elimination of the toxic compound gossypol from cottonseeds by Sunilkumar et al. (2006). This study clearly demonstrated the feasibility of a targeted RNAi-based approach to solve an age-old problem of cottonseed toxicity and provided an avenue to exploit the considerable quantities of protein and oil available in the global cottonseed output. Gossypol and related terpenoids are present throughout the cotton plant in the glands of foliage, floral organs, and bolls, as well as in the roots. These terpenoid compounds protect the cotton plants from both insects and pathogens and are essential for the survival of cotton under normal agricultural conditions, where it is exposed to a variety of pests and diseases, although its presence in the seed might be expendable (Townsend and Llewellyn 2007). A glanded-plant and glandless-seed trait does occur naturally in the native Australian cotton species *Gossypium sturtianum*. Gossypol-filled glands develop as the seeds germinate in order to provide the needed protection against pests and pathogens. Efforts to breed this trait into cultivated cotton were not successful, mainly due to considerable genome differences between two species. A natural glandless mutant of cotton was identified in the 1950s, and several breeding programs were launched to transfer this glandless trait into commercial cotton cultivars. However, glandless cotton varieties were a commercial failure due to their extraordinary susceptibility to insect pests since they constitutively lacked gossypol and protective terpenoids.

Remarkably, this long-standing goal of cotton geneticists was achieved through RNAi-mediated silencing that eliminates toxic gossypol from cottonseeds. This objective was achieved by silencing the δ-Candinene synthase. This enzyme catalyzes the first committed step involving in the cyclization of farnesyl diphosphate to δ-candinene (Chen et al. 1995), the compound from which gossypol and other sesquiterpenoid compounds are derived. The δ-cadinene synthase gene was silenced under the control of the cottonseed-specific $\propto$-globulin promoter. Seed from the transgenic cotton plants exhibited a significant reduction in gossypol content, whereas the cotton foliage, floral parts, and floral organs contained normal levels of gossypol. Transgenic plants with seed gossypol levels reduced to as low as 99% were stable and were maintained for three generations (Sunilkumar et al. 2006). Gossypol values in the seeds from some of the silenced lines were well below the limit deemed safe for human consumption by the United Nations Food and Agriculture Organization and World Health Organization. In this example, once again the use of an endogenous gene and a native promoter of cotton ensure appropriate spatial and temporal expression of the transgene.

6.3.2.3 Tearless Onions

Manipulation of plant secondary metabolic pathways can result in dramatic and simultaneous down- and up-regulation of products within that pathway and the even production of novel products. Onions *(Allium cepa*) synthesize a unique set of secondary sulfur metabolites. When onions are chopped, these metabolites are cleaved by the enzyme alliinase into their corresponding sulfenic acids and volatile sulfur compounds that give the respective flavors. One of the volatiles released is Lachrymatory Factor (LF), the chemical responsible for inducing tearing. In addition, it is hypothesized that LF production causes the absence of otherwise predicted sulfur volatiles, analogs of which in garlic (*A. sativum*) are known for their health attributes (Eady et al. 2008). Current "tearless" onion cultivars (e.g., Vidalia) are achieved through deficient uptake and partitioning of sulfur and/or growth in sulfur-deficient soils, but in so doing they accumulate fewer secondary sulfur compounds in the bulb, reducing their sensory and health qualities compared with more pungent high-sulfur cultivars.

Eady et al. (2008) made a healthier and tearless onion by reducing the levels of Lachrymatory Factor Synthase (LFS) and preventing the conversion of 1-propenyl sulfenic acid to the undesirable and irritating LF. By means of RNAi, LFS activity in onions was reduced by up to 1,544-fold. When these onions are chopped, significantly reduced levels of LF are produced. No phenotypic abnormalities were reported among the transgenic onion plants compared to the controls in greenhouse experiments. The authors also confirmed that RNAi silencing of LFS shifted sulfur metabolism away from tearing agents, giving rise to a cascade of predicted secondary compounds that had not been detected previously or only in trace amounts in onion (Minorsky 2008). The researchers hope to initiate formal taste evaluation trials of these transgenic, tearless onions following regulatory approval. These onions may add potential value to the future agrifood industry due to their desirable health promoting attributes.

6.3.2.4 Low-acrylamide French Fries and Potato Chips

Acrylamide is a toxic substance that is naturally produced in starchy foods as a result of high-temperature cooking, such as baking, grilling, or frying. In 2002, the Swedish National Food Administration reported alarmingly high levels of acrylamide in carbohydrate-rich heated foods (products from potato tubers, wheat flour, and coffee beans) (Tareke et al. 2002). Since acrylamide is considered as probably carcinogen for animals and humans, this finding resulted in worldwide concern. In potato, acrylamide is formed by a Maillard-type reaction among amino acids (Asparagine) and reducing sugars at high frying temperatures (Mottram et al. 2002). Several food companies recently have agreed to substantially reduce the acrylamide levels in fried potato products over the next 3–5 years. Thus novel methods to reduce the acrylamide levels in fried potato products have been a major goal for potato industry breeding programs.

Previous attempts to lower the acrylamide levels negatively affected color, texture, taste, and overall consumer palatability of the fried products. In some cases these required changes in current grower or processor practices, which limited their broader acceptance (Rommens et al. 2008). A most effective approach to reduce acrylamide levels by RNAi technology has recently been reported by Simplot^{TM} Company (Rommens et al. 2008). Potato plants were transformed with an all-native sequence RNAi-silencing construct (Rommens 2004; Yan et al. 2006) that targets two asparagine synthetase genes under the control of potato tuber specific promoters. Asparagine synthetase catalyzes the ATP-dependent conversion of aspartate into asparagines. The resulted intragenic plants produced tubers with very low levels of the acrylamide precursor asparagine. Green house experiments have shown that these lines contained a 20-fold reduction of asparagines in tubers. Chips and fries processed from these tubers remarkably showed as little as 5% accumulation of acrylamide compared to the controls. Surprisingly, this modification neither altered overall yield of the tubers grown under greenhouse conditions; nor the color, texture, or taste of the fried products. If silenced lines retain all the original agronomic characteristics under field conditions, the researchers hope all-native-DNA potato products with very low levels of acrylamide could be offered as a choice on the market.

6.4 Limitations of RNAi-Silencing Technology

A possible limitation of RNAi technology is the off-target effects of siRNA that might silence nontarget genes. Since RNAi is based on sequence recognition, targeting a gene by RNAi may give rise to the silencing of another gene that has short regions of similar sequence. This phenomenon is referred to as off-target silencing. However, no potential off-target effects were reported so far in plants (Mansoor et al. 2006; Xu et al. 2006). Transcript profiling has extensively been used in plant research and as of yet no off-target expression level changes have been noticed. Several reports recently confirm that RNAi in plants exhibits a high level of sequence specificity. Nevertheless, the possibility of off-target effects in plants cannot be ruled out and therefore needs careful attention. Caution is warranted in interpreting gene function and phenotype information resulting from RNAi experiments.

Other limitations of RNAi occur when there is a lack of efficacy or variable levels of silencing effects. Traditional DNA mutations (insertion or deletion) most often are irreversible and the effect on the function of the affected gene is generally predictable. By contrast, RNAi silencing can have widely varying effects depending on the target gene and the region of the transcript that is targeted. Sibling plants carrying identical RNAi constructs can produce varying phenotypes (Wang et al. 2005; Small 2007). There are multiple reasons for this variability that need to be considered when interpreting RNAi phenotypes. One should examine multiple independent lines to check for a reproducible phenotype and attempts should

also be made to check that off-target effects are not affecting genes related to the target gene.

6.5 Future Directions

Only a few of the achievements through RNAi technology were discussed in this chapter. However, RNAi has been used for a variety of applications, including altering the flower color or obtaining novel colors by RNAi-mediated engineering of flavonoid biosynthetic pathways (Tanaka et al. 2005); developing cyanogen-free transgenic cassava, which is a major staple crop in sub-Saharan Africa (Jorgensen et al. 2005); and changing the pattern and quality of fatty acid composition of soybean by silencing the undesirable fatty acids (Flores et al. 2008). These examples, among others, demonstrate that targeted gene silencing can be used to modulate biosynthetic pathways in a specific tissue in order to obtain a desired phenotype, a feat that is often not possible by traditional breeding. These studies open the gateway to new frontiers in the use of genetic manipulation to enhance global food supply. Over the horizon, plant molecular biologists and plant breeders can see the possibility of using similar approaches to eliminate harmful compounds from plants that otherwise could serve as potential food sources, such as *Lathyrus sativus*, a hardy tropical/subtropical legume plant that naturally contains the neurotoxin β-*N*-oxalylamino-L-alanine (BOAA). This noneconomic crop is a potential target of RNAi silencing of the gene(s) responsible for the production of BOAA. A similar strategy could be applied to fava beans in order to eliminate various glycosides, undesirable compounds for human consumption. Tissue- or organ-specific silencing approaches are needed to achieve targeted gene silencing in particular plant cells with minimal interference to the normal plant lifecycle (Tang and Galili 2004).

The study of gene silencing has led to a revolution in the understanding of gene expression, as underlined by the recent award of a Nobel Prize on this concept (2006 Nobel Prize in Physiology/Medicine to Dr. Andrew Fire and Dr. Craig Mello on Gene Silencing). Because RNAi is a very efficient knockdown technology in plants, it is useful for genetic improvement in cultivars and crop plants with low transformation efficiencies, although transformation is still a challenge to face. That having been said, RNAi has clear advantages over insertional mutagenesis. The primary advantage is an ability to specifically target the gene of interest. As RNAi is a homology-dependent process, careful selection of a unique region of the target sequence can ensure that a specific gene family member is silenced, or targeting highly conserved sequence domains can silence multiple members of a gene family. In this way, redundancy is not limiting. RNAi can also be used to analyze the functions of essential genes. Variable levels of gene silencing can be achieved in different transgenic lines using the same RNAi construct, allowing selection of lines with a greater or lesser degree of silencing.

Since its identification several years ago, RNAi has become the technology of choice for plant scientists investigating gene function and manipulating plants for novel traits. Though most of the products developed using RNAi technologies are yet to hit the commercial line, there seems to be an enormous promise for future improvement especially quality traits in crop plants. The use of tissue-specific and inducible promoters should improve our ability to silence gene expression in desired target tissues and at the appropriate developmental stage, thus minimizing off-target effects. New approaches such as amiRNA (Warthmann et al. 2008) promise to bring more precision and predictability to the technology in the near future. Nevertheless, the bottleneck of public acceptance of crops derived through genetic modification should not be neglected, and remains a political and not technical challenge.

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Chapter 7
Transgene Integration, Expression and Stability in Plants: Strategies for Improvements

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7.1 Introduction

The transfer of DNA into plants has been common practice for over 20 years, and transgenic plants are now a burgeoning industry. In 2007, over 114 million ha (282.4 million acres) of transgenic crops were grown commercially in 23 countries, the most prevalent traits being herbicide tolerance, pest resistance, or both traits stacked together (James 2007). In the laboratory, one encounters a vastly greater diversity of traits, including disease resistance, stress tolerance, nutritional improvement, modified development, and the use of plants to produce specific, high-value molecules, such as secondary metabolites, chemical precursors, antibodies, vaccine subunits, and industrial enzymes. It is notable that in the majority of cases, the purpose of gene transfer into plants is to achieve a specific, desirable phenotype. Plants that fail to live up to expectations are routinely discarded so that the best performers can be nurtured.

Despite the focus on phenotype, over the last decade there has been an increasing interest in creating transgenic plants to study the process of gene transfer itself (Kohli et al. 2003). On the academic side, it has been appreciated for many years that the structure of a transgene locus can have a major influence on the level and stability of transgene expression; thus, researchers have studied DNA integration mechanisms, particularly with regard to how transgenes interact with the plant's DNA repair and genome defense systems. On the applied side, the global adoption of transgenic crops and the development of transgenic plants producing pharmaceuticals and other important molecules have attracted the interest of regulatory authorities (Ramessar et al. 2008). The demand for robust risk assessment practices

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means that transgenic plants have to be characterized in great detail, including information on the sequence, structure, organization, and genomic position of the transgenic locus. Recently, this has culminated in the first report of the genome sequence of a transgenic plant, including the analysis of the transgenic locus (Ming et al. 2008). The principles and practices of transgenic technology have come under scrutiny, leading to research focusing on the use and elimination of marker genes, the role of vector sequences that integrate along with the transgene, and the random nature of transgene integration events with regard to copy number, transgene orientation, and transgene rearrangements. Researchers, therefore, have practical as well as academic reasons for studying transgene integration and expression, and have developed new ways to analyze transgenic loci. Current research focuses on ways to better control the way DNA integrates into the plant genome.

In this chapter, we describe the methods used to study transgene locus structure and discuss evidence supporting current models of transgene integration for both *Agrobacterium*-mediated transformation and direct transfer methods. We discuss how transgene loci are organized and how this affects the level and stability of transgene expression from generation to generation. Finally, we look to the future by describing how recent research has advanced the state of the art in gene transfer technology.

7.2 Methods for the Analysis of Transgenic Loci

Most gene transfer experiments are phenotype driven, by which we mean that successfully transformed plants tend to be identified on the basis of the phenotype conferred by the transgene rather than the structure of the transgene itself. This is pertinent because the appearance of the desired phenotype is prima facie evidence that the transgene has integrated into the genome and is intact, thus allowing expression of the encoded protein. Since most transgenic plants are regenerated under selection for the product of a selectable marker gene, the fact that a transgenic plant exists at all indicates that at least one intact copy of the marker gene is present in the genome. Similarly, the phenotypes conferred by any other transgenes can be used as evidence to support successful integration and expression. This information is of limited value, however, because it divides all plants into just two categories – (a) plants transformed with at least one intact transgene and (b) plants not transformed at all or transformed with a nonfunctional transgene. It provides no quantitative information, yet every gene transfer experiment produces a population of plants with a range of phenotypes reflecting the level of transgene expression. Since the same input DNA is used in each case, the only explanation for quantitative differences in phenotype is differences in the structure and activity of the integrated transgenes.

The technique used most commonly for a definitive analysis of transgenic loci is the Southern blot, in which genomic DNA is digested with one or more restriction

enzymes, fractionated by agarose gel electrophoresis, denatured, transferred to a membrane, and hybridized to a labeled probe. Many different types of information can be obtained from Southern blots depending on the restriction enzymes and probes used. One of the most common strategies is to use an enzyme that cuts once within the transgene in combination with a probe that hybridizes to the body of the transgene. This generates DNA fragments whose size depends on the distance between a fixed point in the transgene (the restriction site) and the adjacent restriction site in the genomic DNA, which of course varies according to the site of insertion. Where multiple copies of a transgene have integrated, a single cutter enzyme tends to generate a unique pattern of bands that serves as a genetic fingerprint of that plant and all its descendants, thereby helping to identify clonal relatives of the original transformant and allowing transgene segregation to be followed through generations. Because it is unlikely that any of the transgene copies will generate identical-sized bands (unless they are perfect concatemers, in which case the band size will correspond exactly to the size of the transgene), this method also provides an estimate of transgene copy number. A variant of the technique is to use a probe that hybridizes to the vector backbone instead of the transgene body, which helps to identify inserts of vector DNA.

Another handy method is to use an enzyme that cuts twice in the transgene and liberates a specific DNA cassette in combination with a probe that hybridizes to that cassette. If all copies of the transgene are intact, there should be only one hybridizing band, corresponding to the size of the cassette, and the intensity of the hybridization signal will be proportional to the number of transgene copies (since one cassette should be released from each integrated transgene copy). Copy number determination is best achieved by "spiking" genomic DNA from an untransformed plant with a known amount of transforming plasmid DNA, and then digesting this and the genomic DNA from genuine transgenic plants. It may be useful to set up a series of control DNA samples, containing for example one, five, and ten copies of the transforming plasmid per genome equivalent of DNA. In this way, a calibration curve of signal intensities relative to copy number can be generated, onto which any transgenic plant can be mapped. Band sizes greater or smaller than the diagnostic fragment indicate truncations or rearrangements. It is also useful to digest genomic DNA with an enzyme known not to cut within the transgene. If all copies of the transgene have integrated at a single locus as a concatemer, digestion with such enzymes should liberate the locus as a single, high-molecular-weight fragment. Thus, the presence of two or more bands suggests either the presence of two or more independent transgenic loci (this can be confirmed by segregation analysis as discussed below), or the presence of interspersed genomic DNA between transgene copies at a single locus (this can be confirmed, if required, by fiber-FISH as discussed below).

The polymerase chain reaction (PCR) is a rapid technique that can be used to confirm transgene integration through the use of primer combinations that generate a transgene-specific product. Although quicker than Southern blots and more

amenable to multiplexing, false positives can occur through the amplification of episomal plasmid DNA so the PCR should only be used indicatively, with Southern blots used for definitive confirmation of DNA integration. Long PCR is a variation that allows larger products to be amplified and is potentially useful for analyzing larger transgenic loci (Mehlo et al. 2000). Another PCR variant, real-time quantitative PCR, is now used for the rapid estimation of transgene copy number by comparison with a control sample in which a single-copy endogenous gene is amplified. The relative signal intensities of the control and transgenic samples reveal the transgene copy numbers in the transgenic plants (Li et al. 2004; Yang et al. 2005). Although the PCR can show the presence or absence of a transgene and provide a dependable copy number estimate, it provides little in the way of information about the structure of a transgenic locus unless the genomic flanking sequences are already known. DNA sequencing is the highest-resolution transgene analysis method, and permits the precise definition of structural organization and rearrangement. It also allows the nature of transgene-genomic and transgene-transgene junctions to be investigated at the nucleotide level, and the integration site in genomic DNA to be identified. Prior to sequencing, the transgenic locus must be isolated, which can be achieved by standard molecular cloning or through a variety of methods such as inverse PCR, thermal asymmetric interlaced (TAIL)-PCR, or plasmid rescue (Twyman and Kohli 2003). The complete sequencing of a transgenic papaya variety, SUNUP, was recently reported, allowing intricate structural analysis of the transgenic locus (Ming et al. 2008).

All these methods involve the identification of discrete DNA fragments of precise length, which is useful for fine structural analysis but not for the characterization of transgenic loci at the chromosome level. In this context, fluorescence in situ hybridization (FISH) can be useful as it allows target sequences to be identified in isolated DNA fibers, interphase chromatin, and even metaphase chromosomes. FISH involves the use of fluorescently labeled nucleic acid probes to identify particular target sequences, revealing higher-order transgene organization and the distribution of integration sites. FISH to metaphase chromosomes allows the insertion sites to be mapped cytogenetically and simultaneous analysis in interphase allows the nuclear territory of transgenes to be determined (Abranches et al. 2000). Fiber-FISH on extended chromatin gives an overview of locus structure, revealing the presence of single-copy inserts, transgene concatemers, and interspersed genomic DNA (Jackson et al. 2001). The resolution of FISH lies somewhere between that of genetic segregation and Southern blot hybridization and can provide important correlative data for both techniques.

Segregation analysis involves studying the transmission of particular DNA sequences or the phenotypes thus conferred over several generations of transgenic plants. Closely linked transgene copies are unlikely to be separated by recombination, while widely separated loci are likely to segregate at meiosis in some plants. This allows the number of transgenic loci to be determined. Problems with phenotype analysis include the misleading results caused by epigenetic gene silencing, but analysis of DNA sequence segregation by Southern blot hybridization can be highly informative.

7.3 Locus Structure in Plants Transformed by *Agrobacterium tumefaciens*

7.3.1 Principles of Gene Transfer

Agrobacterium tumefaciens is a soil pathogen that colonizes wounded plant cells and induces the formation of a tumor (or crown gall) that produces special amino acid derivatives called opines, which the bacteria are able to use as a carbon and nitrogen source. The ability of virulent *Agrobacterium* strains to induce tumor growth and opine synthesis, and the capacity to utilize opines, is conferred by a resident tumor-inducing plasmid (Ti-plasmid). During the colonization process, a segment of DNA from this plasmid called the transferred DNA (T-DNA) is transferred to the plant nuclear genome. The T-DNA encodes enzymes that synthesize auxins and cytokinins, resulting in unregulated cell proliferation, and enzymes that synthesize opines from standard amino acids (reviewed by Gelvin 2003).

The Ti plasmid is a naturally occurring vector for plant transformation, but wild-type Ti-plasmids are not suitable vectors for genetic engineering in plants because they are too big to manipulate, and the oncogenes contained in the T-DNA cause uncontrolled proliferation of transformed plant cells and prevent efficient regeneration. The T-DNA must therefore be moved to a smaller, more convenient vector, and disarmed by deleting the oncogenes. A marker gene must also be included to allow transformed cells to be propagated. T-DNA transfer is controlled by about 30 genes located in a separate virulence (*vir*) region of the Ti plasmid, and these must be supplied in trans using a binary vector system if the T-DNA is placed on a smaller plasmid. Modern binary vectors contain multiple unique cloning sites within the T-DNA, a *lac*Z marker gene for blue-white selection of recombinants, and a choice of selectable markers to identify transformed plant cells (Hellens et al. 2000).

The transfer mechanism is pertinent to the resulting locus structure. The T-DNA is flanked by 25-bp imperfect direct repeats known as border sequences, which are not transferred to the plant genome intact, but they are required for the transfer process. T-DNA transfer is mediated by the *vir*A and *vir*G gene products, which transduce external signals and activate other *vir* genes resulting in the construction of a pilus for DNA transfer, and the release of the T-DNA by an endonuclease comprising the products of the *vir*D1 and *vir*D2 genes. This introduces either single-strand nicks or a double-strand break at the 25-bp borders of the T-DNA. It is thought that the intermediate formed (a double stranded T-DNA or a single T-strand) may depend on the virulence functions particular to the *Agrobacterium* strain (Steck 1997). Either the left or right border sequence can initiate T-DNA transfer, although it is more usual for initiation to occur at the right border due to the presence of an adjacent overdrive sequence, which is recognized by the VirC1 and VirC2 proteins and acts as a transfer enhancer (Shaw et al. 1984). For this reason, deletion of the right T-DNA border severely reduces the efficiency of transfer, whereas deletion of the left border has little effect (Jen and Chilton 1986).

The VirD2 protein remains covalently attached to the 5′ end of the processed T-DNA strand and has been proposed to protect the T-DNA against nucleases, to target the DNA to the plant cell nucleus, and to help integrate it into the plant genome (Tzfira et al. 2000).

7.3.2 T-DNA Locus Structure

Most investigations of T-DNA transfer have suggested that there may be preferential integration into transcription units, with up to 90% of events occurring in genes (e.g., Lindsey et al. 1993). In petunia, FISH analysis showed that T-DNA inserts were found preferentially at the gene-dense distal chromosome sites (Wang et al. 1995; Ten Hoopen et al. 1996), and a comparative analysis in *Arabidopsis* and rice showed that T-DNA inserted randomly in the *Arabidopsis* genome (which is globally gene-rich, with little repetitive DNA) but homed in on the 10–20% of the rice genome known to be gene-dense while avoiding the more widespread heterochromatic regions (Barakat et al. 2000). It has also been suggested that T-DNA integration occurs preferentially in regions showing microhomology to the T-DNA borders (Matsumoto et al. 1990), which may also be enriched in the transcribed part of the genome. The bias in T-DNA insertion is valuable for gene-tagging experiments, ensuring a high gene hit rate in genomes with large tracts of gene-poor heterochromatin while showing little bias among different genes (Jeon et al. 2000; Weigel et al. 2000; Hsing et al. 2007; Wan et al. 2009).

The structure and complexity of transgenic loci generated by *Agrobacterium* depends on the strain, plant species, and explant type, but generally gives rise to lower transgene copy numbers than direct transformation methods. An informative experiment was performed by Cheng et al. (1997) by transforming wheat using both *Agrobacterium* and particle bombardment. Of 26 *Agrobacterium*-mediated transformants, more than one-third contained a single T-DNA insert, half contained 2–3 copies, and the remainder (about 15%) contained 4–5 copies. There were no transformants containing more than five T-DNAs. In contrast, from the population of 77 bombarded transformants, only 13 (17%) contained a single copy of the transgene. The maximum number of transgene copies in this population was not reported. Hu et al. (2003) also observed more complex transgene insertions from particle bombardment than from *Agrobacterium*-mediated techniques. More recently, similar experiments in barley showed that all the *Agrobacterium*-derived lines contained 1–3 copies of the transgene, while 60% of the transgenic lines derived by particle bombardment contained more than eight copies (Travella et al. 2005). Dai et al. (2001) found in rice that the average transgene copy numbers were 1.8 for *Agrobacterium*-derived lines and 2.7 for plants obtained by particle bombardment. However, Khanna and Raina (2002) observed multiple transgene insertions in rice transformants generated through both techniques together with the transfer of partial T-DNA fragments.

The organization of integrated T-DNA sequences differs among *Agrobacterium* strains, but a common feature of nopaline-type derivatives such as C58 is the preferential integration of T-DNA as dimers with an inverted repeat configuration, linked either at the left or right borders (Jones et al. 1987; Jorgensen et al. 1987). Where cotransformation is carried out with two T-DNAs containing different markers, the different T-DNAs were often present as heterodimer inverted repeats, preferentially around the right border (De Block and Debrouwer 1991). Similarly, cotransformation of rice with the vectors pGreen and pSoup (each containing different selectable and visible markers) resulted in 56% of plants with the two T-DNAs cointegrated, although there was also a high proportion of plants containing separate integration events (Afolabi et al. 2004). In contrast, Spielmann and Simpson (1986) carried out transformation using the octopine *Agrobacterium* strain LBA4404. They found only two integration events among the 22 characterized transformants that resulted in dimer formation, while most of the rest were single-copy integrations. When cotransformation experiments were carried out with this strain (McKnight et al. 1987), three double transformants were obtained and in all cases the two T-DNAs were genetically unlinked. These results suggest that the virulence functions carried by a particular *Agrobacterium* strain strongly influence the structure of the transgene locus.

Another important aspect of locus structure is the amount and types of transgene rearrangement. Occasionally, it has been reported that T-DNA has undergone spontaneous rearrangement prior to or during integration (e.g., Offringa et al. 1990; Puchta et al. 1992), and this has been demonstrated directly by fiber-FISH in potato (Wolters et al. 1998). In some cases, rearrangements may be induced by specific recombinogenic sequences such as the CaMV 35S promoter (Kohli et al. 1999), which may have been responsible for T-DNA rearrangements in some transgenic potato lines (Porsch et al. 1998). In many cases, however, rearrangements may reflect "collateral damage" occurring spontaneously during the transfer process. Afolabi et al. (2004) found that nonintact T-DNAs were present in >70% of transgenic rice lines, in most cases reflecting loss of the mid to right border portion of the T-DNA. Similarly, Rai et al. (2007) found that about 50% of rice plants transformed with a T-DNA containing the phytoene synthase (*psy*) and phytoene desaturase (*crt*I) genes showed evidence of T-DNA rearrangements, and in the majority of cases the rearrangements occurred in the *crt*I expression cassette, which was adjacent to the right T-DNA border.

7.3.3 T-DNA Integration Mechanism

A number of groups have investigated the structure of genomic/T-DNA and T-DNA/T-DNA junctions in plants and have concluded that integration occurs by illegitimate recombination (see Salomon and Puchta 1998; Somers and Makarevistch 2004). A strand invasion mechanism of integration has been proposed (reviewed by Tinland 1996), in which the 3′ end of the T-strand initiates the

integration process by hybridizing to a short region of homology in the plant genome, the second strand being completed by primer extension of the plant DNA. Other models suggest conversion of the T-strand into a double-stranded intermediate, which integrates at the site of naturally occurring chromosome breaks via double-strand DNA break repair. This is supported by experiments that show transformation efficiency increases following UV irradiation, which generates nicks and breaks in genomic DNA. However, since T-DNA integration occurs normally, if less frequently, in DNA repair mutants, it is possible both mechanisms occur simultaneously.

DNA repair models argue that proteins encoded by the host plant have a much more important role in T-DNA integration than *Agrobacterium* proteins, such as VirD2, which are imported into the plant with the T-DNA. However, since VirD2 protein remains covalently attached to the 5′ end of the T-strand during transfer it is also likely to influence integration (Ward and Barnes 1988). In an in vitro assay, VirD2 can ligate together a cleaved T-DNA border sequence but cannot ligate T-DNA to other genomic targets unless plant cell extracts are also present (Pansegrau et al. 1993; Ziemienowicz et al. 2000).

Plant proteins are certainly required for integration, as a number of *Arabidopsis* mutants have been identified that are deficient for T-DNA insertion. The role of DNA strand break repair in T-DNA integration was supported by the discovery of *Arabidopsis* mutants *uvh1* and *rad5*, which are hypersensitive to UV and gamma irradiation, respectively, and show a low frequency of stable transformation by *Agrobacterium*. Since these mutants showed normal levels of transient expression, it was suggested that they caused deficiencies in the repair of radiation-induced breaks and that break repair is essential for T-DNA integration (Sonti et al. 1995). However, Nam et al. (1998) showed that *uvh1* is no less transformation proficient than wild-type plants and that *rad5* is deficient for both transient and stable transformation, indicating that the dysfunction affects a process occurring much earlier than T-DNA integration. Other mutants resistant to *Agrobacterium* transformation (*rat* mutants) have been identified, and five are thought to be blocked at the point of T-DNA integration (Nam et al. 1999). One of the corresponding genes, *rat5*, encodes a histone protein, suggesting that efficient T-DNA integration is dependent on chromatin structure at the integration site.

Much can be learned about the T-DNA integration mechanism by the inspection of borders, especially the borders between adjacent T-DNA sequences in multicopy insertions. The formation of heterodimers during cotransformation argues in favor of T-DNA concatemerization prior to integration. Although inverted repeats around the right border are often precise, those around the left border and those separating direct T-DNA repeats are often characterized by the insertion of variable-sized regions of filler DNA, which may be derived from the T-DNA sequence or from plant genomic DNA (De Buck et al. 1999; Kumar and Fladung 2000, 2002). This suggests either the simultaneous integration of multiple T-DNAs at a single locus, or a two-phase mechanism, in which a primary T-DNA integration event stimulates further secondary integrations in the same area, similar to those proposed for particle bombardment (see Sect. 7.4.3). Zhu et al. (2006) carried out a

comprehensive study of T-DNA border characteristics in a population of transgenic rice plants including 156 T-DNA/genomic DNA junctions, 69 T-DNA/T-DNA junctions, and 11 T-DNA/vector backbone junctions, which included 171 left borders and 134 right borders. Conserved cleavage was observed in 6% of left and 43% of right borders, microhomology was observed in 58% of T-DNA/genomic DNA, 43% of T-DNA/T-DNA, and 82% of T-DNA/vector junctions, mostly at left borders, and about one-third of the T-DNA/genomic DNA and T-DNA/T-DNA junctions showed evidence of filler DNA (up to 344 bp). This was derived mainly from the T-DNA region adjacent to the breakpoint and/or from the rice genomic DNA flanking the T-DNA integration site, with T-DNA/T-DNA filler DNA showing the greatest complexity. Interestingly, when two T-DNAs were integrated in the inverted repeat configuration, significant truncation was always observed in one of the two T-DNAs, whereas with direct repeat configuration, large truncations were rare. These data suggested no single integration mechanism could account for all observations, but the presence of filler DNA at many of the junctions argued that a template-driven DNA synthesis mechanism must be involved, probably reflecting abortive gap repair through a synthesis-dependent strand annealing (SDSA) process. For example, a 16-bp filler DNA that was identical to a reversed T-DNA fragment close to the right border was observed at a left/right border junction. This was most likely produced by invasion of the 3′ end of a T-DNA into another T-DNA near the right border in reverse orientation during recombination or interaction of these two T-DNAs. When the right border is not protected by VirD2, it is subjected to 5′ exonuclease degradation that creates a free 3′ end in its complementary strand. This 3′ end is able to invade another template to produce filler DNA at the right border end. Multiple template switches can be used to explain the origin of complex filler DNA structures, and longer regions of homologous DNA might reflect a single-strand annealing process in addition to SDSA.

7.3.4 Cotransfer of Vector Backbone Sequences

Agrobacterium was initially thought to be a clean transformation method because the T-DNA is more or less precisely defined (cleavage occurs at a precise position within the right border repeat and the cleavage site at the left border varies by about 100 bp). However, it is now evident that T-DNA transfer is much less precise than originally envisaged, and 25–30% of transformants may commonly contain vector sequences linked to the T-DNA insert, indicating that the cleavage reaction during T-DNA transfer can be rather inefficient (Martineau et al. 1994; Rai et al. 2007). Other studies have shown that in some systems, the frequency of vector backbone transfer can reach as high as 66% (Afolabi et al. 2004). Ramanathan and Veluthambi (1995) constructed binary vectors, in which the selectable marker was located outside the left T-DNA border. In accordance with the T-DNA transfer mechanisms discussed above, it was considered likely that this strategy would catch those transfer events in which transfer, initiated at the right border, overran the left

border and terminated somewhere along the plasmid backbone. Surprisingly, these investigators found that none of the transformants contained any T-DNA sequences, indicating that, in these cases, transfer had initiated at the left border and had proceeded around the plasmid away from the T-DNA, presumably breaking off before completing the circuit and reaching the right border sequence. Further investigations have shown that vector sequence transfer is probably a very common event, occasionally involving the entire plasmid backbone with or without the T-DNA. Concatemers of the entire binary vector have also been seen, indicating that transfer does not necessarily terminate at the T-DNA border even after one or more complete circuits of the vector (Wenck et al. 1997). The exact structure of the insert and the presence or absence of T-DNA in recovered transgenic plants depend of course on the position of the selectable marker. In the strategy of Ramanathan and Veluthambi (1995), the external position of the marker allowed non-T-DNA transformants to be recovered. The experiments carried out by Kononov et al. (1997) are particularly informative because this group constructed binary vectors, in which a selectable marker was present within the T-DNA and a screenable marker gene was present outside either the left or the right borders of the T-DNA. Over 200 transformants were obtained under selection and 75% were shown to carry the external screenable marker gene *gus*A. Interestingly, both vectors appeared to transfer *gus*A to the plant genome with equal efficiency, suggesting that T-DNA transfer could be initiated nonselectively at either the left or right borders. It is also notable that Kononov and colleagues used three alternative *Agrobacterium* strains: LBA4404, GV3101, and EHA105, representing octopine, nopaline, and agropine-type virulence functions, respectively. There were no significant differences among the strains in terms of the frequency of vector sequence transfer. Finally, these investigators reported that they could also detect independent integration events involving plasmid backbone sequences alone. Since the selectable marker in these experiments was located within the T-DNA, such vector-only integrations must have occurred in addition to the T-DNA-linked integration events. This indicates that in the natural course of transformation, many vector-only integration events may occur, but will not be recovered under selection. It is also likely that vector-only integration events occur, undetected, in many plant transformation experiments.

7.4 Locus Structure in Plants Transformed by Direct DNA Transfer

7.4.1 Principles of Gene Transfer

A number of direct DNA transfer methods have been developed to transform plants recalcitrant to *Agrobacterium*-mediated transformation (reviewed by Twyman et al. 2002). Among these methods, particle bombardment has become the most successful

because it is based on purely mechanical principles and is therefore not dependent on the biological factors that restrict the *Agrobacterium* "host range". Particle bombardment works with any plant species, variety, and explant, leaving the regeneration of fertile plants rather than the DNA transfer process itself as the only significant bottleneck (Altpeter et al. 2005). Particle bombardment involves the acceleration of small DNA-coated metal particles (either gold or tungsten) into plant tissue with sufficient force to break through the cell wall and membrane. Some of the particles reach the nucleus, where the DNA is released, probably by a simple diffusion mechanism (Altpeter et al. 2005). Notably, the foreign DNA entering a bombarded cell is naked, double-stranded, and competent for both transient episomal expression and integration into the genome. Transient expression also occurs in the process of *Agrobacterium*-mediated transformation, but the T-strand must first be converted into a double-stranded intermediate (Narasimhulu et al. 1996). Other direct DNA transfer methods are gentler, using chemicals (e.g., PEG, calcium phosphate) or physical methods (e.g., electroporation) to persuade plant protoplasts to take up DNA from the surroundings. However, this DNA must ultimately find its way to the nucleus, and integration occurs in the same way as described below for particle bombardment.

While *Agrobacterium*-mediated transformation involves a number of virulence gene products that must be supplied either on the same plasmid as the T-DNA or on a separate binary vector, particle bombardment has no such requirements because the introduction of DNA is governed entirely by external physical factors (Sanford et al. 1993). For convenience, therefore, vectors used for direct transfer are generally based on bacterial cloning plasmids, and incorporate a selectable marker and origin of replication functional in bacteria. In *Agrobacterium*-mediated transformation, the T-DNA is meant to be excised from the vector during the transformation process, and any vector backbone transfer results from inefficient processing. In contrast, there is no such processing in particle bombardment, although this can be achieved before transformation by excising the linear cassette, purifying it, and using just this cassette as the substrate for coating the metal particles (Fu et al. 2000). This practice has the interesting side effect of reducing the complexity of transgene loci as discussed in Sect. 7.4.3.

7.4.2 Transgenic Locus Structure

There have been few studies, in which integration sites generated by particle bombardment have been carefully mapped, so whether there is a preference for inserting in transcription units is not so clear as in the case of T-DNA integration. The variable nature of the input DNA linear cassette sequences should remove any sequence-dependent bias (compared to the preserved ends of the T-DNA), but as discussed in Sect. 7.3.4, the T-DNA cutting process can overshoot the left and/or right border, so it is likely that the substrates for integration are equally variable in T-DNA transfer. Chen et al. (1998) noted that in rice plants cotransformed with up

to 13 plasmids, there was no preference for the integration of particular transgenes, indicating that the insertion mechanisms operated independent of input gene sequence. Svitashev et al. (2000) showed by FISH analysis of transgenic oat that integration occurred randomly with respect to the A/D and C genomes, and there was no preference for chromosomes from a particular genome. However, the majority of integration events occurred at telomeric and subtelomeric regions, which are typically gene-rich. It is also possible that this preferential integration may reflect some aspect of the nuclear architecture in oat rather than the distribution of genes, since FISH analysis of a limited number of transgenic wheat plants generated by particle bombardment showed no preferential integration in terms of the chromosome region. In the commercial papaya variety SUNUP, five of the six sequences flanking the three indentified transgene integration sites were genomic copies of plastid genes (Ming et al. 2008). Since the plastid genome is more AT-rich than typical genomic DNA, this both supports the possibility of preferential insertion in or near genes and matches the observation of AT-rich sequences at the insertion sites in other transgenic lines generated by *Agrobacterium* and direct DNA transfer.

Unlike the situation with *Agrobacterium*-mediated transformation, a vast literature has accumulated on the structure and complexity of transgenic loci generated by direct DNA transfer, particularly particle bombardment. As discussed in Sect. 7.3.2, T-DNA integration usually occurs with a low copy number, rarely exceeding five copies, and the T-DNA is generally intact. In contrast, direct DNA transfer often generates much larger transgenic loci. Typically, these contain from 1 to 20 transgene copies (e.g., Klein et al. 1987; Register et al. 1994; Cooley et al. 1995; Dai et al. 2001; Travella et al. 2005). The structure of such loci is highly variable, comprising single copies, tandem or inverted repeats, concatemers, intact transgenes, truncated and rearranged sequences, and interspersed genomic DNA. The analysis of transgenic cereal plants by FISH to extended DNA fibers, metaphase chromosomes, and interphase chromatin has revealed a higher-order level of organization where discrete integration events are interspersed by large fragments of genomic DNA, up to several hundred kilobase pairs in length. This organization, which generates immense (megabase) transgenic loci, appears unique to particle bombardment, and could thus reflect the nature of the transformation process itself (see Sect. 7.4.3).

A useful overview of transgene organization in wheat has been reported by Jackson et al. (2001) using the technique of fiber-FISH. This study showed that transgene loci in bombarded wheat plants can be organized in three ways. The simplest arrangement, described as a type III locus, is characterized by a single discrete fiber-FISH signal corresponding approximately to the length of the transforming plasmid. This represents an intact, single copy transgene. Type III loci may be present uniquely in a given plant, or there may be two or more unlinked inserts representing multiple genetic loci. These two possibilities can be distinguished by FISH to metaphase chromosomes and genetic segregation analysis. Other loci, described as type I loci, are longer than the single plasmid copy yet still generate a continuous signal along the extended chromatin fiber. For example, Jackson and colleagues reported a type I transgenic locus with a continuous signal of 77 kb,

representing 11 contiguous plasmid copies. Such loci represent concatemers of the transforming plasmid and are characterized by the absence of intervening genomic DNA. The presence of concatemers can also be confirmed by Southern blot analysis and sequencing across plasmid/plasmid junctions. Loci thus characterized have been described by Kohli et al. (2003) as "transgene arrays" (Fig. 7.1). Until the late 1990s, both head-to-head and head-to-tail concatemers had been sporadically reported in the literature, but it was unusual for the structure of a transgenic locus to be examined in such detail. Concatemerization is probably quite a common phenomenon. Extensive concatemerization, for example, has been reported by Hadi et al. (1996) in transgenic soybean simultaneously transformed with 12 different plasmid vectors. The remaining class of locus (type II) is the most complex. It is characterized by fiber-FISH signals that extend for a significant distance (>100 kb) over the chromosome, but which are punctuated regularly by intervening segments

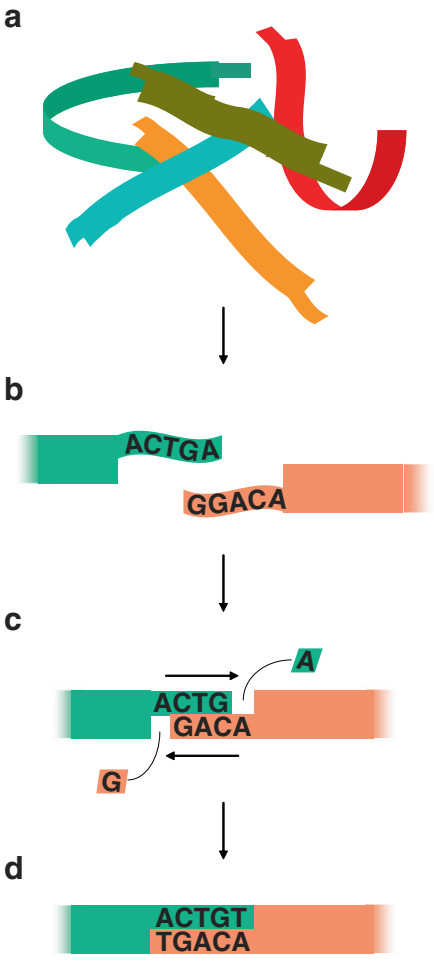

Fig. 7.1 Mechanism for transgene integration at regions of microhomology. A mixture of DNA fragments with ragged ends (**a**) interacts with a double-stranded DNA break with partially complementary ragged ends (**b**). Repair synthesis across the gap (**c**) generates a recombination junction (**d**) which may be completely conserved if the homology is precise, or may involve either the loss of terminal sequences or the insertion of filler DNA if the homology is partial

of genomic DNA (no signals). Such loci have also been identified in transgenic oat, rice, barley, and maize. In barley, for example, some transgene integration sites showed simple structures represented by one single FISH signal, whereas in others it was possible to identify up to six spots organized in a linked cluster and separated by barley DNA, making the locus several megabase pairs long (Travella et al. 2005). Kohli et al. (2003) defined such loci as "transgene clusters." Type II loci contain genomic interspersions ranging from a few tens of base pairs to approximately 10 kb (Fig. 7.2). Although dispersed over a distance of up to 100 kbp, such

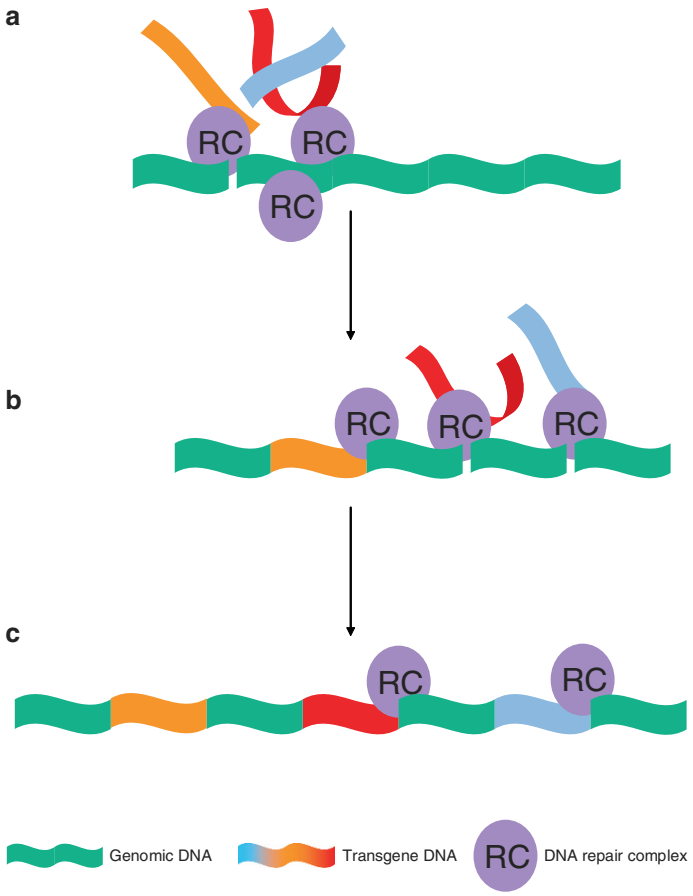

Fig. 7.2 Explanation for the formation of transgene arrays and transgene clusters interspersed with genomic DNA. A mixture of DNA fragments interacts with a double-stranded DNA break where a repair complex has already assembled (**a**). The repair complex may stitch together DNA fragments to form concatemers prior to integration, or may integrate single copies. The first integration event stimulates further repair complex activity nearby, resulting in additional nicks and breaks in the genomic DNA that act as further integration sites (**b**). This results in a cluster of transgenes (single copies and concatemers) interspersed with short regions of genomic DNA (**c**)

loci would still be expected to generate a single discrete signal if FISH analysis was applied to metaphase chromosomes due to the low resolution of this technique. However, the analysis of metaphase wheat chromosomes by FISH has revealed an unexpected third level of organization, involving the dispersion of transgene arrays and/or clusters over a larger area comprising megabase pairs of DNA (Abranches et al. 2000). Instead of discrete spots for each transgenic locus, two or more separable FISH signals were often observed, restricted to a particular chromosome region (Fig. 7.3). To be separable at the cytogenetic level, each signal must be interspersed by hundreds of kilobase pairs of genomic DNA. Similarly large genomic interspersions have been seen in transgenic oat (Svitashev and Somers 2001).

Interestingly, FISH analysis of interphase chromatin and metaphase chromosomes in the same transgenic wheat plants showed that the dispersed metaphase FISH signals could come together at interphase (Abranches et al. 2000). Occasionally, the signals clustered at a specific region of the nucleus but remained discrete.

7.4.3 Mechanisms of Transgene Integration

The analysis of plasmid/plasmid and plasmid/genomic junctions in transgenic plants generated by particle bombardment reveals features characteristic of illegitimate recombination similar to those seen for T-DNA junctions, suggesting that the same overall integration mechanisms may be involved (Svitashev et al. 2002). For example, such junctions are characterized by regions of microhomology, filler DNA, trimming of the DNA ends so sequences are lost and AT-rich elements surrounding the junction site, with similarity to topoisomerase I binding/cleavage sites (Fig. 7.1). In the analysis of multiple plasmid/plasmid junctions in 12 transgenic rice lines, Kohli et al. (1998) observed ten plants with microhomology at the junctions and two plants where junctions appeared to be generated by blunt ligation, with no overlap. A similar ratio of conserved end-joining to microhomology-mediated recombination was observed by Gorbunova and Levy (1997) and Salomon and Puchta (1998). Topoisomerase I sites were also observed adjacent to 10 out of 12 junctions characterized in transgenic *Arabidopsis* plants generated by particle bombardment (Sawasaki et al. 1998) and in four of the six junctions in the commercial SUNUP variety of papaya (Ming et al. 2008). Illegitimate recombination, therefore, appears to be responsible both for the integration of foreign DNA into the plant genome and the linking of multiple plasmid copies, which is similar to the mechanism proposed for T-DNA integration (Sect. 7.3.3).

Any model for transgene integration following particle bombardment must take into account the three-tier organization revealed in transgenic cereals: contiguous arrays, interspersed clusters, and widely dispersed FISH signals. Two-phase transgene integration mechanisms have been proposed to explain the first two levels of organization, and in such models concatemerization is proposed to occur prior to integration, while interspersion occurs during the integration process (Kohli et al.

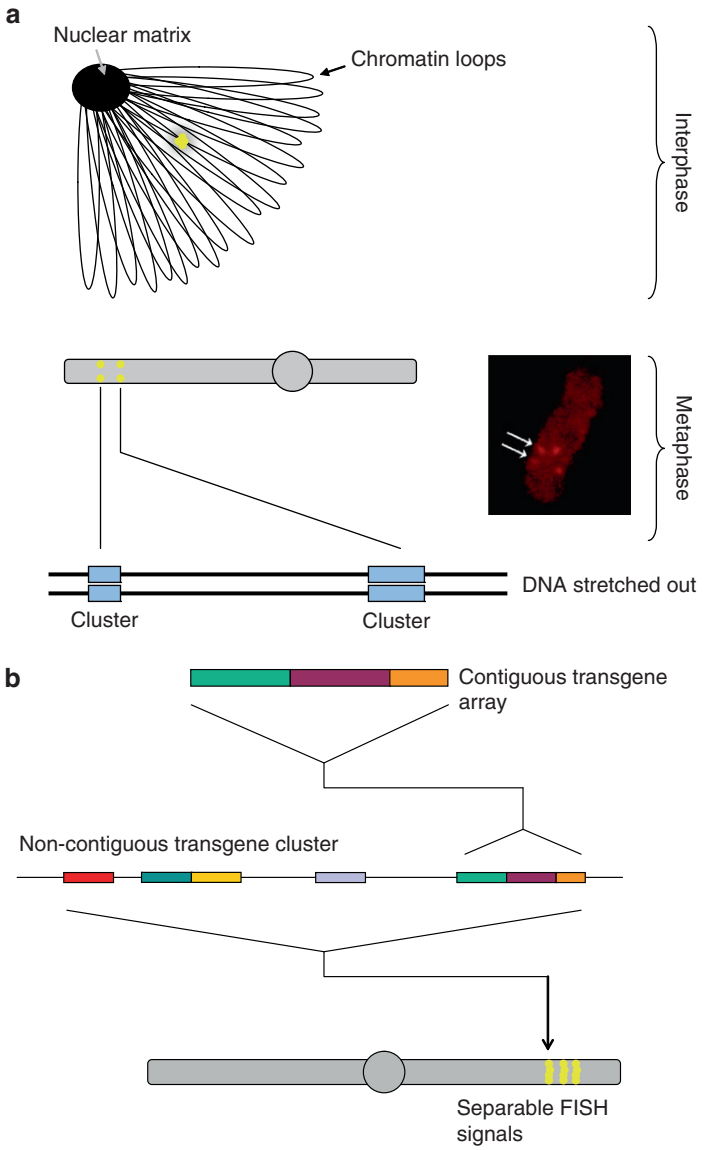

Fig. 7.3 Higher order transgene locus organization in cereals transformed by particle bombardment. Transformation occurs during interphase, when the chromatin is distributed into specific nuclear zones and territories. If a metal particle causes localized damage, DNA repair complexes will form at these sites and initiate transgene integration (**a**). During metaphase, when FISH analysis is generally carried out, loci that are brought together in interphase may be separated, resulting in multiple signals from the same transformation event (**b**). If the DNA were stretched out, this would reveal large (megabase) interspersed sequences, which have also been observed in fiber-FISH experiments

1998; Pawlowski and Somers 1998; Svitashev et al. 2002) (Fig. 7.2). In each model, penetration of the cell is proposed to elicit a wound response, which would include the induction of DNA repair enzymes, such as nucleases and ligases. The presence of these enzymes and an excess of foreign DNA would result in the linking together of several copies to form concatemers, which would be the substrates for integration. This might be stimulated by homology between individual copies of transforming plasmids, and "backbone" homology might also result in the concatemerization of plasmids carrying different transgenes in cotransformation experiments. However, cotransformation and cointegration were also shown to occur when two nonhomologous minimal cassettes were used for transformation, so homology might not be as important as the presence of free DNA ends (Fu et al. 2000). Kohli et al. (1998) suggested that transgene clusters arise in a second phase where a primary integration event occurring by illegitimate recombination at a chromosome break generates a "hot-spot" for further integration events in the same area. This might be due, for example, to the presence of local repair complexes that slide along the DNA and introduce nicks which can be exploited by more foreign DNA (Gelvin 1998). Pawlowski and Somers (1998) suggested an alternative second phase where a number of discrete transgene concatemers integrate simultaneously at a site containing multiple replication forks. Although there is no direct evidence for either mechanism, it is interesting to note that DNA integration is stimulated in rapidly dividing cells and is blocked in *Arabidopsis* mutants lacking essential components of the DNA recombination machinery.

The higher order organization of transgenic loci observed by metaphase FISH is thus far unique to particle bombardment and demands a model which takes into account the three-dimensional structure of the nucleus. In one scenario, it is possible that the transformation event affects a local region of the interphase nucleus. For example, it is possible that the metal particle causes damage to a particular area of chromatin, which is arranged in loops attached to the nuclear matrix. If the particle "skims" several loops, there will be regions of DNA damage close together in trans, but widely separated in the cis configuration were the DNA to be stretched out (Fig. 7.3). Each of these sites could act as a nucleation point where foreign DNA diffusing from the metal particle is used to patch up double-strand breaks, generating widely separated arrays and/or clusters (Abranches et al. 2000; Kohli et al. 2003). In support of this induced break and repair model, Svitashev et al. (2000) have shown that in six of 25 transgenic oat plants generated by particle bombardment, transgene integration sites were associated with rearranged chromosomes. This suggests that DNA breaks caused by incoming particles are repaired with foreign DNA and may also result in deletions, inversions, and translocations involving genomic DNA. Chromosomal rearrangements have also occasionally been seen associated with T-DNA integration (Nacry et al. 1998; Laufs et al. 1999).

The model above suggests that dispersed metaphase signals come together at interphase due to the physical position of the transgenic loci at the moment of transformation. In another scenario, the bringing together of transgene sites at interphase could represent recruitment, for example to a common transcription

factory in the nucleus (Cook 1999). A further scenario involves transgenes that are brought together by virtue of their homology, perhaps as a consequence of their initial placing in the same region of the nucleus. This is an exciting prospect because the coincidence of FISH signals observed in wheat nuclei could represent a physical basis of the postulated DNA-DNA interactions that precede transcriptional transgene silencing in plants (see below).

Transgene rearrangements following particle bombardment have been widely reported in the literature, and many publications repeat the "lore" that direct DNA transfer is more likely than T-DNA transfer to generate complex rearranged loci. The number of rearrangements that can be detected depends entirely on the resolution of the method being used. Thus, careful analysis of locus structure by Southern blot hybridization, PCR, and DNA sequencing has recently shown that rearrangements may be more widespread than first envisaged in both transformation methods. The analysis of transgenic oat loci by Somers and colleagues has shown that transgene rearrangements can be extensive and extremely complex, with multiple small insertions, inversions, and deletions within any transgene, plus the presence of filler DNA (Svitashev et al. 2000). In maize, Mehlo et al. (2000) noted that every single plant among the population they analyzed showed some form of rearrangement, and they speculated that undetected "minor" rearrangements could be responsible for many instances of transgene silencing otherwise attributed to epigenetic effects (see Sect. 7.5). In particular, certain transgene rearrangements were not detectable by Southern blot hybridization because they were too subtle, but they could be picked up by PCR and sequencing. Since in most cases, Southern blot hybridization is used to determine whether a given locus is intact or rearranged, this suggests caution should be used in relying on such results, since only "major" rearrangements can be detected in this manner.

Few researchers have characterized transgene rearrangements in detail, but work by Kohli et al. (1999) has shown that rearrangements may involve palindromic sequences in the transforming plasmid, which tend for form three-dimensional structures such as hairpins and cruciforms. These investigators characterized 12 transgenic rice lines, transformed by particle bombardment, which had been shown to contain rearranged transgenes. Interestingly, they found that an imperfect palindrome in the CaMV 35S promoter was involved in one-third of all rearrangements, i.e., the sequence of this palindrome was adjacent to the rearrangement junction. Similar phenomena have been noted in T-DNA transformants containing the same promoter (Sect. 7.3.2). This sequence has the ability to adopt a cruciform secondary structure, which may stimulate recombination events. Many other promoters contain palindromic sequences of variable length within 100 bp of the transcription start site. The DNA secondary structures formed at these sites enable DNA-protein interactions for transcription under normal circumstances, but may also participate in aberrant recombination events. The fully sequenced papaya genome (Ming et al. 2008) also revealed a number of previously unidentified transgene rearrangements, i.e., a 1,533-bp fragment composed of a truncated, nonfunctional *tet*A gene and flanking vector backbone sequence, and a 290-bp nonfunctional fragment of the *npt*II gene, in addition to the intact, primary transgene conferring virus resistance.

7.5 Locus Structure and Transgene Stability

One of the most profound insights to come from the detailed analysis of transgene loci over the last decade is that many integrated transgenes contain minor rearrangements. As discussed above, these are difficult to pinpoint using low-resolution detection methods such as Southern blot hybridization and FISH, but high-resolution methods such as sequencing are rarely used as a routine analysis tool. Therefore, the impact of physical rearrangements on transgene expression is likely to be vastly underestimated, since unstable loci are often blamed on epigenetic phenomena with no further analysis to draw confirmatory evidence.

There are many factors that influence transgene stability, and these lead to highly variable expression within populations of plants generated in the same gene transfer experiment. One of the most important factors is the position effect, which reflects the influence of genomic DNA surrounding the site of transgene integration (Wilson et al. 1990). Another is the structure of the locus, including the number of transgene copies, their intactness, and their relative arrangement, which influences the likelihood of physical interactions and further recombination within the locus (physical instability) and the induction of silencing through DNA methylation and/or the production of aberrant RNA species from the locus (Heinrichs 2008).

7.5.1 Position Effects

Specific position effects result from the influence of local regulatory elements on the transgene. For example, an integrated transgene may come under the influence of a nearby enhancer, such that its expression profile is modified. The effect is transgene-specific because the enhancer interacts with regulatory elements in the transformation construct to control transcription; hence, the final expression pattern reflects the combined influence of both regulatory elements. Such effects are clearly revealed by entrapment constructs, which contain minimal control sequences linked to a visible marker gene and therefore "report" the activity of local regulatory elements (e.g., Goldsbrough and Bevan 1991).

As well as specific position effects governed by local regulatory elements, nonspecific position effects can also be generated by the surrounding chromatin architecture. Where the local environment is favorable for transgene expression, i.e., a positive position effect, it is generally taken for granted. However, nonspecific and repressive position effects reflect the integration of the transgene into a chromosomal region containing repressed chromatin (heterochromatin). The molecular features of heterochromatin, including its characteristic nucleosome structure, deacetylated histones, and hypermethylated DNA, spread into the transgene causing it to be inactivated (Pikaart et al. 1998). Analysis of the genomic context of silenced transgenes suggests that integration in the vicinity of certain repetitive DNA sequences, such as microsatellites and retrotransposon remnants, may predispose the transgene to silencing (Tanako et al. 1997). The chromosomal

location is important, since in many plants, the genes are restricted to a small portion of the genome known as gene space, and the majority of the DNA is taken up by repetitive sequences. Thus, stable transgene expression has been associated with gene-rich telomeric and subtelomeric integration sites, whereas mosaic expression and silencing occurs at predominantly heterochromatic centromeric loci.

A third type of position effect reflects the tolerance of the surrounding DNA for "invasion" by foreign DNA. In this case, the effect is not automatic (as above) but is set off by the presence of the transgene. It appears that certain sequences can trigger de novo methylation, perhaps because the GC-content or sequence architecture is recognized as abnormal (reviewed by Kumpatla et al. 1998). Prokaryotic DNA may be recognized in this manner, since silencing is often associated with the presence of prokaryotic vector backbone DNA, particular binary vector sequences joining T-DNA to genomic DNA (Iglesias et al. 1997).

7.5.2 Locus Structure Effects

At least three aspects of locus structure influence transgene stability and expression: copy number, intactness, and arrangement. It is natural to assume that increasing the number of copies of a particular transgene will lead to an increase in the level of its product. However, even from the earliest plant transformation experiments, it was appreciated that multiple transgene copies could induce transgene silencing and that the phenomenon was associated with DNA methylation at the transgenic locus (e.g., Gelvin et al. 1983; Hepburn et al. 1983). A strikingly visual demonstration of this effect was provided by introducing the maize *AI* gene into mutant petunia plants with white flowers. Expression of the transgene resulted in pelargonidin production, generating a red pigment. However, it was shown that red flowers generally appeared on plants with single copy transgenes, while plants with multiple transgene copies had white or variegated flowers. Where transgene silencing had occurred, increased methylation of the transgene DNA was observed (Meyer et al. 1992). Similarly, it was thought that the amount of pigment in wild-type petunia flowers could be increased by introducing extra copies of the chalcone synthase (*chs*) gene (Napoli et al. 1990). Chalcone synthase converts coumaryol-CoA and 3-malonyl-CoA into chalcone, a precursor of anthocyanin pigments. The presence of multiple transgene copies was expected to increase the level of enzyme and hence cause stronger flower pigmentation. However, in about 50% of the plants recovered from the experiment, exactly the opposite effect was observed. The flowers were either pure white, or variegated with purple and white sectors. It appeared that integration of multiple copies of the transgene led not only to the suppression of transgene expression, but also to the cosuppression of the homologous endogenous gene.

Rooke et al. (2003) looked at the integration, inheritance, and expression of transgenes in six transgenic wheat lines generated by particle bombardment with two plasmids containing genes encoding a glutelin subunit and a selectable marker, respectively. Transgene insertion number ranged from 1 to 15, with most lines carrying multiple copies consistent with previous reports (Becker et al. 1994;

Blechl and Anderson 1996; Srivastava et al. 1996; Stoger et al. 1998; Cannell et al. 1999). Four of the transgenic loci were clusters interspersed with genomic DNA, in some cases enough to allow independent segregation which contrasts with previous reports in which cointegration and cosegregation were the norm, and independent segregation rare (Stoger et al. 1998). There was no evidence for a direct correlation between transgene copy number and expression level, and no evidence for cosuppression of endogenous glutelin genes even in multicopy lines. The presence of multiple transgene copies has been implicated in transgene silencing, but other studies in cereals have shown that multiple copies do not necessarily lead to silencing and can even enhance expression levels in proportion to copy number (Stoger et al. 1998; Gahakwa et al. 2000). In contrast, Spencer et al. (1992) failed to recover progeny expressing the marker transgenes from maize lines containing more than five or six copies of the integrated plasmid, while Cannell et al. (1999) observed silencing or a gradual reduction in marker gene expression over three generations of transgenic wheat lines. It has been suggested that the production of lines with single transgenes or low copy numbers is desirable as such lines may be more stable and less likely to exhibit transgene silencing (Finnegan and McElroy 1994), but with several studies reporting contrary results, this may indicate that chance plays a role in the impact of copy number, perhaps reflecting insertion events near to boundary elements (Sect. 7.5.3).

Variation in transgene expression levels can also result from uncontrolled differences in experimental protocols reflecting gene-environment interactions, which means that proper comparisons between transgenic lines should take place in a standardized environment. To study sources of spurious variation, transgene expression levels were quantified over five homozygous generations in two independent transgenic rice lines created by particle bombardment (James et al. 2004a, b). Both lines contained the same *gus*A expression unit which was stably inherited, and all plants were cultured and sampled using previously developed standardized protocols. Plants representative of each generation (T_2–T_6) were grown either all together or across several different growth periods. Where the plants were grown and characterized independently, the amount of extraneous variation in transgene expression levels was up to three-fold higher than in plants grown and analyzed together. This study therefore provided important evidence that the growth and analysis of all plants from all generations together, using standard operating procedures (SOP), can reduce extraneous variation associated with transgene expression and is the key to improving the reproducibility of transgenic studies conducted over multiple generations (James et al. 2004a, b).

7.5.3 Overcoming Position and Locus Structure Effects by Buffering the Transgene

As discussed earlier, analysis of the genomic context of transgene integration sites has shown that silenced transgenes are often surrounded by repetitive elements, which are sequestered into repressed chromatin. The same studies have also shown

that stably expressed transgenes are often associated with matrix attachment regions (MARs) (Iglesias et al. 1997). MARS are AT-rich elements that attach chromatin to the nuclear matrix and organize it into topologically isolated loops (Holmes-Davis and Comai 1998). A number of highly expressed endogenous plant genes have also been shown to be flanked by matrix attachment regions (e.g., Chinn and Comai 1996). One strategy that has been proposed to overcome position effects is therefore to protect or buffer the transgene by flanking it with MARs prior to transformation. In this way, it is hoped that the transgene will form a discrete chromatin loop which will be isolated from surrounding chromatin.

Several experiments have been carried out in which a reporter gene such as *gus*A has been flanked by MARs. Such constructs have been introduced into transgenic plants and compared to populations containing the same reporter gene without MARs (e.g., Mlynarova et al. 1994, 1995, 1996; Van Leeuwen et al. 2001; Mlynárová et al. 2002). Generally MARs do have a positive effect on transgene expression and can significantly reduce position effects, but they cannot rescue all lines and restore full expression. It is acceptable to say that they generally reduce expression variability within a population (e.g., Breyne et al. 1992). Expression may increase as much as five-fold, but some remarkable exceptions include a 25-fold enhancement using a yeast MAR and a 140-fold enhancement using a tobacco MAR in tobacco callus (Allen et al. 1993, 1996).

7.5.4 *Overcoming Position and Locus Structure Effects by Homologous Recombination*

As discussed in Sects 7.3.3 and 7.4.3, transgene integration in higher plants occurs almost universally by illegitimate recombination, which may involve microhomology but is not dependent upon it. Since there is only minimal sequence relationship between the transgene and the genomic region into which it integrates, the experimenter has little control over the integration site. In other systems, notably yeast, homologous recombination is favored over illegitimate recombination if the vector carries a homology region that matches the yeast genome, allowing endogenous genes to be altered by gene targeting (Schiestl and Petes 1991). In the context of controlling transgene integration, it also allows transgenes to be inserted at specific loci, a strategy that should allow favorable sites for transgene integration to be chosen, theoretically abolishing position effects and reducing the complexity of locus structure.

Although widely used in microbial systems, homologous recombination occurs with a very low efficiency in plants (illegitimate recombination occurs about 10^5 times more frequently than homologous recombination, making genuine targeting events difficult to isolate). Only one plant species has been shown to undergo efficient nuclear homologous recombination (the moss *Physcomitrella patens*) and the results in higher plants have been much less impressive, with targeting efficiencies as low as 10^{-6} (Lee et al. 1990; Offringa et al. 1990; Miao and Lam 1995; Risseeuw et al. 1995, 1997; Kempin et al. 1997; Reiss et al. 2000; Hanin et al. 2001).

A transgene has also been repaired by homologous recombination in tobacco (Paszkowski et al. 1988). More recently, promising results have been achieved using a T-DNA-mediated gene-targeting strategy involving a long homology region in combination with a strong counterselectable marker in rice (Terada et al. 2002). Targeting frequencies of up to 1% have been achieved using this system (reviewed by Ida and Terada 2004 and Cotsaftis and Guiderdoni 2005). Gene targeting has also been reported recently in maize (D'Halluin et al. 2008).

There has also been interest in the use of zinc-finger endonucleases to make targeted double-strand breaks in the plant genome, so that homologous recombination is favored at such sites (Kumar et al. 2005). The modular nature of zinc-finger transcription factors means that recombinant DNA technology can be used to "mix and match" these DNA-binding domains to create recombinant proteins with unique sequence specificities. Zinc fingers are motifs approximately 30 amino acids in length which coordinate a Zn^{2+} ion and bind to DNA sequences three base pairs long. Combining different zinc fingers in series allows proteins to be tailor made to bind longer DNA sequences. When a nonspecific DNA endonuclease is incorporated into such a protein, it becomes a targeted DNA cutting tool (Lloyd et al. 2005; Wright et al. 2005; Zeevi et al. 2008; Cai et al. 2009).

7.5.5 Overcoming Position and Locus Structure Effects by Organelle Transformation

The plant cell contains not only a nuclear genome, but also organellar genomes in the chloroplasts and mitochondria. The chloroplast is a useful target for gene transfer because tens of thousands of chloroplasts may be present in a single plant cell, and each chloroplast may contain multiple copies of its chromosome. Genetic engineering of the plastid genome offers several advantages over nuclear transformation including that integration occurs by homologous recombination, the high copy number of transgenes in a homoplasmic cell, and the absence of gene silencing phenomena due to the lack of position and locus structure effects, and the absence of DNA methylation in the plastid genome (Daniell et al. 2005; Daniell 2006; Bock 2007). The recombination machinery is very active in chloroplasts and can induce rearrangements, as observed in some of the first tobacco transformants generated with the *aad*A selectable marker (Svab and Maliga 1993). The stability of a plastid transgene has been evaluated in soybean transformants over six generations. These transformants had integrated the *aad*A selection cassette in the intergenic region between the *rps*12/7 and *trn*V genes. Three independent homoplasmic T_0 transformation events were selected and ten plants from each event propagated to generation T_5 in the absence of selection pressure. Neither transgene rearrangement nor wild-type plastids were detected in generation T_5 by Southern blot analysis. All tested progenies were uniformly resistant to spectinomycin. Therefore, soybean transformants of generations T_0 and T_5 appear to be genetically and phenotypically identical (Dufourmantel et al. 2006).

7.5.6 *Overcoming Position and Locus Structure Effects by Site-specific Recombination*

Site-specific recombination is a form of recombination that occurs at short, specific recognition sites rather than DNA sequences with long regions of homology but no particular sequence specificity, as is the case for homologous recombination. Site-specific recombination is not ubiquitous – indeed different organisms encode their own very specific systems that include the cis-acting recombinogenic sites and the enzymes that recognize them and carry out the recombination event. Therefore, the target sites for site-specific recombination can be introduced easily and unobtrusively into transgenes, but recombination will only occur in a heterologous cell if a source of the specific recombinase enzyme is also supplied. As with the homologous recombination strategy discussed above, position and locus structure effects can be eliminated by introducing foreign DNA at a specific, favorable locus. A number of different site-specific recombination systems have been identified and several have been studied in detail (Sadowski 1993). The most extensively used are Cre recombinase from bacteriophage P1 (Lewandoski and Martin 1997) and FLP recombinase from the yeast *Saccharomyces cerevisiae* (Buchholz et al. 1998). These have been shown to function in many heterologous eukaryotic systems including transgenic plants (Metzger and Feil 1999). Both recombinases recognize 34-bp sites (*lox*P and *FRP*, respectively) comprising a pair of 13-bp inverted repeats surrounding an 8-bp central sequence. *FRP* possesses an additional copy of the 13-bp repeat sequence, although this is nonessential for recombination.

The Cre-*lox*P system has been used most widely in plants, often for controlled transgene excision (particularly selectable marker genes after transformation) but more recently for controlled transgene insertion (Gilbertson 2003; Lyznik et al. 2003; Puchta 2003; Marjanac et al. 2008). Marker genes are usually excised in the T_1 generation once transgene expression is verified, allowing the separately introduced *cre* gene to segregate in T_2 plants. This method has been used in many crops including wheat (Srivastava and Ow 2001, 2003), maize (Kerbach et al. 2005; Djukanovic et al. 2006; Hu et al. 2006; Vega et al. 2007, 2008), rice (Chen et al. 2004; Srivastava et al. 2004; Chawla et al. 2006; Moore and Srivastava 2006; Vega et al. 2008), potato (Kopertekh et al. 2004a,b), and tomato (Gidoni et al. 2003; Coppoolse et al. 2005). Controlled integration has been studied in transgenic plants already engineered to contain recipient *lox*P sites (Srivastava et al. 2004). In this study, three different recipient wheat lines were generated by bombarding plants with the *lox*P sequence, and these were subsequently bombarded with a *gus*A construct also containing flanking *lox*P sequences and a *cre* gene. Following transformation, about 80% of lines contained *gus*A at the recipient site, many with single-copy transgenes and others with concatemers. Both types of locus were stably inherited. There was much less variation in expression among the single copy lines (Srivastava et al. 2004).

Chawla et al. (2006) generated 18 different transgenic rice lines containing a precise single copy of *gus*A at a designated site. In seven of these lines, additional

copies of the transgene integrated at random sites by illegitimate recombination, while 11 showed "clean" integration by site-specific recombination only. The single-copy lines were stable over at least four generations and showed consistent levels of expression, which doubled in homozygous plants. In contrast, the multi-copy lines showed variable expression and some fell victim to transgene silencing. Interestingly, where the site-specific and illegitimate integration loci segregated in later generations, transgene expression was reactivated in the plants carrying the site-specific integration site alone, whereas close linkage between the site-specific and random integration prevented segregation in other lines and the silencing persisted.

An exciting recent development is the GENE DELETOR system, which is a hybrid of the Cre-*lox*P and FLP-*FRT* systems. The GENE DELETOR is based on a fusion recognition site (*lox*P-*FRT*), which is inefficient when both recombinases are expressed but highly efficient when either one of the recombinases is expressed alone, giving up to 100% efficiency in populations of up to 25,000 T_1 transgenic plants (Luo et al. 2007).

Another use for Cre-*lox*P is the simplification of locus structure by resolving multicopy loci to a single transgene copy (Srivastava et al. 1999). A strategy was developed in which the transformation vector contained a transgene flanked by *lox*P sites in an inverted orientation. Regardless of the number of copies integrated between the outermost transgenes, recombination between the outermost sites resolved the integrated molecules into a single copy. The principle was proven by resolving four multicopy loci successfully into single-copy transgenes.

7.5.7 Overcoming Position and Locus Structure Effects Using Minichromosomes

In bacteria, plasmid vectors are maintained as episomal replicons to make cloning and isolating recombinant DNA a simple procedure. When it comes to expressing heterologous genes in eukaryotic cells, episomal vectors are widely used to avoid position effects, hence the development of yeast episomal vectors, yeast artificial chromosomes, mammalian plasmid vectors carrying virus origins of replication (e.g., SV40-based vectors, herpesvirus-based vectors), and plant expression vectors based on plant viruses (all of which replicate episomally). The yeast artificial chromosome system is the most relevant in this context because it allows genes of any size to be introduced into the yeast genome as an independent replicating unit that is treated by the cell as an additional chromosome. YACs comprise a yeast centromere and telomeres, the origin of replication (autonomous replicating sequence) and selectable markers. More recently, analogous systems have been developed to maintain genes as episomal minichromosomes in plants. These have many advantages for plant genetic engineering including the ability to express large transgenes or groups of transgenes, and the ability to rapidly introduce new linkage groups into diverse germplasm.

Carlson et al. (2007) created plant minichromosomes by combining the *DsRed* and *npt*II marker genes with 7–190 kb of maize genomic DNA fragments containing satellites, retroelements, and other repeat sequences commonly found in centromeres. The circular constructs were introduced into embryogenic maize tissue by particle bombardment and transformed cells were regenerated and propagated for several generations without selection. The minichromosomes were maintained as extrachromosomal replicons through mitosis and meiosis, and showed roughly Mendelian segregation ratios (93% transmission as a disome with 100% expected, 39% transmission as a monosome crossed to wild type with 50% expected, and 59% transmission in self-crosses with 75% expected). The *DsRed* reporter gene was expressed over four generations, and Southern blot analysis indicated the genes were intact.

7.6 Epigenetic Silencing Phenomena Resulting From Complex Locus Structures and High-Level Expression

As stated earlier, the earliest plant transformation experiments showed that multiple transgene copies could induce transgene silencing, in some cases associated with the cosuppression of homologous endogenes. Transgene silencing can occur through two overlapping pathways, one acting at the transcriptional level (characterized by the reduction or abolition of transcription from one or more copies of the transgene) and one acting post-transcriptionally (transcription from the silenced locus is required to initiate silencing) (Hammond et al. 2001). Transcriptional silencing is often correlated with increased methylation in the promoter regions of affected loci, and both the methylation and the silencing tend to be heritable through meiosis. Post-transcriptional silencing requires homology in the transcribed regions, which may become methylated, and the silencing effect can be reset at meiosis. Post-transcriptional silencing is also known as RNA silencing.

Transcriptional silencing occurs when transgene repeats somehow act as a trigger for de novo DNA methylation. It has been shown that inverted repeats can form secondary structures that are favored substrates for methylation, and thus it is likely that cis DNA-DNA pairing may be involved in such processes. However, transgene silencing can also occur in trans, i.e., silencing interactions may occur between unlinked loci. This has been shown, for example, in sequential transformation events with homologous transgenes, or where two plant lines carrying homologous transgenes have been crossed (Matzke and Matzke 1990, 1991). In this situation, it is likely that physical interactions between transgenes may occur to mediate silencing, and that DNA methylation may somehow be transferred from one site to another. As discussed in Sect. 7.4.3, FISH studies in transgenic wheat provide tantalizing evidence for such interactions in the interphase nucleus (Abranches et al. 2000). Since the CaMV 35S promoter is frequently used for transgene expression and can form cruciform structures that induce transgene rearrangements (Kohli et al. 1999), it may also play a role in transcriptional

silencing under certain circumstances. Supporting evidence for this has been provided in studies of activation tag lines in which the CaMV 35S enhancer is used as a random insertional mutagen to hyperactivate adjacent genes and generate gain-of-function phenotypes. It has been noted that such screens using T-DNA cassettes containing the enhancer elements from the CaMV 35S promoter return a low frequency of morphological mutants (Chalfun et al. 2003). Detailed analysis revealed a correlation between the number of T-DNA insertion sites, the methylation status of the enhancer sequence and enhancer activity. All plants containing more than a single T-DNA insert were methylated on the enhancer and its activity was reduced, with the amount of methylation and the reduction of enhancer activity correlating with the number of T-DNA copies, particularly those with right border inverted repeats (Chalfun et al. 2003). Even so, methylation was still detected at a lower frequency in plants without right border inverted repeats suggesting other triggers were active in these lines.

A recurring theme in post-transcriptional silencing is the presence of double-stranded RNA. Double-stranded RNA introduced into the plant cell can trigger the catalytic degradation of homologous RNA molecules and the methylation of homologous DNA sequences in the genome (e.g., Tavernarakis et al. 2000). When carried out deliberately through the expression of hairpin RNA constructs, this process (RNA interference, RNAi) is a potent method for silencing individual genes, generating phenocopies of mutant phenotypes (e.g., see McGinnis et al. 2005; Gordon and Waterhouse 2007). It has been suggested that complex multicopy transgenic loci could also generate hairpin dsRNA, e.g., if two transgenes are present as inverted repeats, or if truncation and/or rearrangements (some perhaps undetectable by standard screening methods) generated small, aberrant dsRNA species (Jorgensen et al. 1996; Que et al. 1997; Muskens et al. 2000). Experiments designed to test this hypothesis specifically have shown that inverted repeat T-DNA configurations and arrangements of tandem repeated transgenes may not be sufficient in all cases to trigger transgene silencing (Lechtenberg et al. 2003), whereas many reports show post-transcriptional silencing in plants with intact transgenes. In such cases it has been suggested that the level of transgene expression may be an important trigger, with "runaway expression" resulting in the most potent silencing effects (Lindbo et al. 1993; Vaucheret et al. 1998; Schubert et al. 2004). Experiments comparing the frequency and potency of cosuppression by sense chalcone synthase transgenes driven by different promoters have shown that a strong promoter is required for high-frequency cosuppression of chalcone synthase genes and for the production of the full range of cosuppression phenotypes (Que et al. 1997). Indeed the correlation between transgene copy number and silencing in some systems may reflect the higher expression level in multicopy loci triggering silencing (Schubert et al. 2004) suggesting that transgenic lines escaping this effect may fall below the threshold for triggering silencing (e.g., Stoger et al. 1998).

The expression threshold model accounts for RNA silencing in intact transgenic loci but it is also possible that such loci are prone to silencing because their high expression promotes the formation of more aberrant RNA products than a poorly expressed transgene. If true, the trigger would still be aberrant dsRNA, the same as

produced by complex, rearranged loci, and it should be possible to mitigate the effects and generate plants with extremely high expression levels. Several studies have shown that RNA silencing in transgenic plants is accompanied by the accumulation of incorrectly processed mRNAs (often lacking polyadenylate tails) (e.g., van Eldik et al. 1998; Metzlaff et al. 2000; Wang and Waterhouse 2000; Han and Grierson 2002) and in at least one case it has been shown specifically that tandem repeats can generate small interfering RNAs (Ma and Mitra 2002). Since dsRNA is unlikely to be generated directly from tandem repeats (as opposed to the situation with inverted repeats), the process must involve an RNA-dependent RNA polymerase. In agreement with this, Luo and Chen (2007) found that RNA silencing in transgenic *Arabidopsis* could be induced by three direct repeats of the *gus*A open reading frame, and this was dependent on the RNA-dependent RNA polymerase encoded by *RDR6*. Normal plants transformed with either three tandem copies of *gus*A or a single copy lacking a polyadenylation site were able to silence a normal *gus*A transgene cassette in trans, but there was no silencing in *rdr6* mutants, which also accumulated long RNA molecules corresponding to *gus*A read-through transcripts of various lengths. Therefore, it appears that the read-through of termination sites leading to the production of long RNA products triggers RNA silencing in an RDR6-dependent manner. A further transgenic line containing a *gus*A transgene with two polyadenylation sites produced fewer read-through transcripts, less siRNA, and therefore showed higher levels of GUS activity. Transgene silencing in tandem repeat transgenes may therefore be triggered by a defense mechanism that evolved to reduce errors caused by read-through transcription (Luo and Chen 2007).

7.7 Conclusions

Transgene integration following *Agrobacterium*-mediated transformation and direct DNA transfer occur by very similar mechanisms, involving illegitimate recombination between genomic DNA and invading transgene DNA strands, and the repair of double-stranded breaks in the host genome. There is often microhomology between the recombining partners, although direct blunt end ligation also occurs. Both transformation methods induce a wound response, resulting in the activation of nucleases, ligases, and recombinases in the host cell. The foreign DNA is simultaneously degraded and concatemerized resulting in transgene arrays containing intact and/or truncated and rearranged copies. Several integration events may occur simultaneously at a cluster of replication forks, or a primary integration event may stimulate further integrations in the local area. Regardless of the mechanism, the result is a transgene cluster interspersed with genomic DNA. In the case of particle bombardment, clusters and arrays may be widely dispersed, generating very large transgenic loci. The position of transgene integration is essentially random within the “gene space” of the plant species. The transgene is thus subject to position effects which may influence its expression, resulting in some cases in transcriptional silencing as the new DNA is sequestered into the

surrounding chromatin. The structure of a transgenic locus may also induce silencing via a number of mechanisms. These include de novo DNA methylation in response to DNA-DNA interactions, the expression of aberrant RNA species (particularly small hairpin RNAs) from truncated and rearranged transgenes or partial transgenes, and the expression of aberrant RNA products from inefficiently terminated transcripts. Position effects can be reduced by buffering the transgene with matrix attachment regions or controlling the site of integration through homologous recombination or site-specific recombination. Alternatively, it may be possible to introduce the transgene into the plastid genome, which does not suffer from position effects. More recent developments such as minichromosomes may provide a method to introduce entire linkage groups and maintain them stably and episomally. Site-specific recombination can also be used to simplify locus structure, by reducing the number of repeats, which may help to reduce the likelihood of RNA silencing. Even so, many reports show that high-level transgene expression is possible in plants with multiple transgene copies, suggesting that the overall level of expression may be relevant, i.e., there may be a trigger level at which silencing is induced. This may involve the detection of high levels of transgene mRNA or may simply reflect the greater likelihood of aberrant RNA products being generated as collateral damage. The recent publication of the full draft sequence of the transgenic SUNUP papaya genome shows that the detailed characterization of the transgene sequence and its flanking regions is not an insurmountable obstacle. Perhaps such intensive analysis will, in the future, allow the accurate prediction of transgene behavior and stability in transgenic plants.

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Chapter 8
Organelle Transformations

Anjanabha Bhattacharya

8.1 Introduction

The world population is increasing at a rapid pace. Agricultural production must match population growth in the near seeable future amid fears of climate change. However, complex traits like crop productivity are difficult to manipulate and take time using conventional breeding (Zuker et al. 1998; Mishra and Srivastava 2004). The availability of a limited gene pool and the failure of wide-crosses among crop varieties in conventional breeding have led to the exploitation of genetic transformation in generating high-yielding crop varieties.

Nucleus transformation is the target of choice for the development of transgenic varieties using one of the several techniques of transformation available today. However, the nuclear genome is large and contains several copies of the same gene, presence of introns, cis-elements, and as such. Therefore, unpredictable results are obtained when transgene(s) are integrated in different parts of the nuclear genome because of positional effect, including, but not limited to, gene silencing or lower levels of transgene expression (Kumar et al. 2004) and off-target influence, thus presenting unusual challenges in their commercialization and restricting consumer acceptability. Removing this analogy required researchers to start looking for other organelles in the plant cell, such as plastids and mitochondria, that could be targeted for genetic transformation. The plastids may differentiate to become chloroplast (green pigment storage plastids), chromoplast (pigment storage organs in fruits), elaioplast (lipid storage), or amyloplast (starch storage). Immature plastids are called pro-plastids. The evolutionary lineage suggests that these organelles were primitively free-living prokaryotes (cyanobacteria), and with the evolution of land plants, they began to form a permanent symbiotic relationship with the host cell and

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became a part and parcel of the plant cell (Lopez-Juez and Pyke 2005). Over time, plastids began to lose the vital genes associated with their independent existence and became dependent on the plant cell machinery to evolve and replicate (Pyke 2007; Provorov 2005). Thus, a sort of endosymbiosis developed between chloroplast and the nucleus, which involved import of many proteins from the cytoplasm (Lopez-Juez and Pyke 2005; Bhattacharya et al. 2007). Organelles like chloroplast and mitochondria have low copy number of genes, several origins of replication, and divide independent of the nucleus; thus, addition of multitransgene in the open reading frame (ORF) results in high levels of gene expression. The first reported case of *Chlamydomonas* chloroplast transformation was by Boynton et al. (1988) almost two decades ago, and subsequent reports of tobacco plastid transformation were by Khan and Maliga (1999).

There is also the absence of epigenetic interference with the inserted transgenes (Bock 2007). The plastid transformation increases the precision of genetic engineering by targeted homologous recombination at specific sites. Specific plastid sequences act as flanking regions for the gene of interest and selectable marker, and are targeted to locate specifically homologous region in the plastid genome thus making precise integration at specific location in plastids. The plastids have several origins of replication (Scharff and Koop 2007) of prokaryotic evolution, and this is essential because they do not depend on nuclear division for transcription. This in turn eliminates the problem of gene silencing; plastids were, therefore, identified as the target of choice for crop transformation. Organelle transformation can also be used to harvest any specific trans proteins as they tend to accumulate at very high levels, thus becoming important in pharmaceutical industries (Buhot et al. 2006). Bohne et al. (2007) concluded that mitochondrial genes and substantial part of plasteome are transcribed by related RNA polymerases. There are also reports of using cross species plastids for effective transformation of the most recalcitrant species (Kuchuk et al. 2006).

8.2 Overview of Organelle Transformation

8.2.1 Plastids

Plastids are of prokaryotic evolution and their genome size is 120–180 kb (Wakasugi et al. 2001). They are related to cyanobacteria and now have become an inseparable part of the plant cell. They have small genome size and their specific sequences are targeted (sequence identified from their sequenced genome; Ruhlman et al. 2006) for construction of suitable vectors for genetic transformation aiming at crop improvement and for therapeutic proteins. Moreover, data mining of the plastid genome could help to identify suitable endogenous flanking sequences for construction of plastid vectors as depicted in Fig. 8.1. A generalized organelle transformation strategy commonly in use is explained in Fig. 8.2.

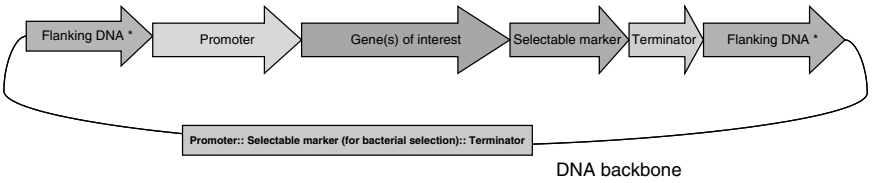

Fig. 8.1 Typical construction of an organelle transformation vector. *Endogenous flanking border sequence homologous to organelle DNA sequence for precise integration and recombination. Such sequences can be indentified by data mining organelle genome sequences

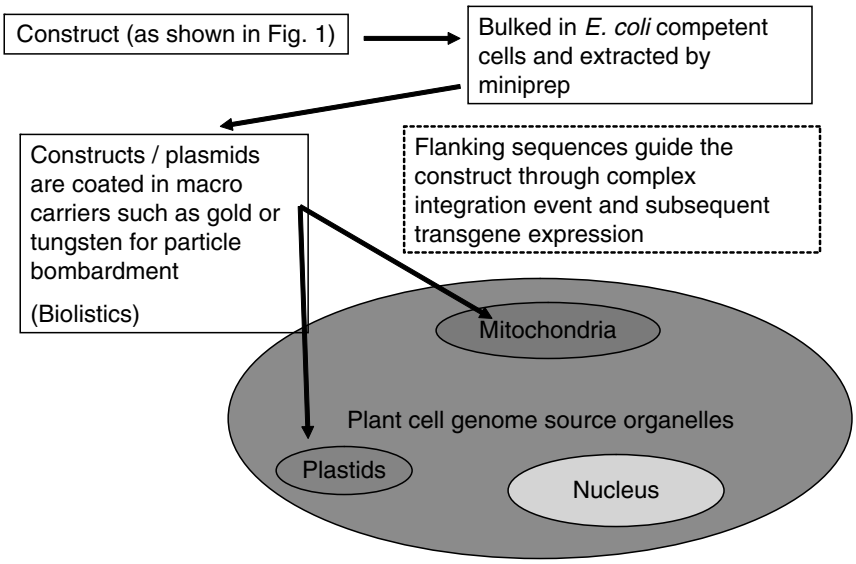

Fig. 8.2 Organelle genetic transformation

The additional advantage of molecular pharming is that the complex enzymes present within the chloroplast forms complex proteins with correct folding thus reducing price incurred for any other additional processing (Koya and Daniell 2005). The plastid acts as a giant factory that produces 100 times more protein than the conventional nuclear transformation (Ishida 2005; Nugent and Joyce 2005). Thus, the pharmaceutical industries are more interested in utilizing these natural bioreactors in plants and reduce cost associated with drug production. Further, the processed protein is free from contaminant, which is a major concern with proteins purified from animal systems. Some successful examples of plastid transformation are listed in Table 8.1. Further, Lutz and Maliga (2007) explained the feasibility of obtaining marker-free transgenic plants with homology based excision, excision by phage recombinase, cointegration of marker gene with the gene of interest.

Table 8.1 Some examples of successful organelle transformation

Plant	Gene introduced	Specific border regions specific for organelle targeting	Organelle involved	Reference
Tobacco	Aminoglycoside 3′-adenyltransferase (*aadA*) [Spectinomycin resistance] and green fluorescent protein (*gfp*)	*trnI* and *trnA*	Chloroplast	Davarpanah et al. (2009)
Tobacco	*HIV Gag (Pr 55)*	chloroplast targeting sequence	Plastids	Scotti et al. (2009)
Sugarbeet	Aminoglycoside 3′-adenyltransferase (*aadA*) and green fluorescent protein (*gfp*)	*rrn16* and *rps12* (intergenic region of plastome)	Chloroplast	De Marchis et al. (2009)
Tobacco and tomato	*HIV* antigen *p24*	p24-Nef fusion gene cassettes	Plastids	Zhou et al. (2008)
Tobacco	*HPV-16 L1* antigen	5′-UTR and *N*-terminal coding segment of a plastid gene, *L1P*, *L1V*	Plastids	Lenzi et al. (2008)
Tomato	Beta-cyclase gene	Intergenic region of plastid gene	Plastids	Wurbs et al. (2007)
Tobacco	Human serum albumin (*HAS*)	Intergenic region of plastid gene	Plastids	Fernáandez-San Millan et al. (2007)
Arabidopsis	*YFP* (Yellow Flurocescent Protein) fused with plastoglobulin 34 (*PGL34*)	Chloroplast targeting sequence	Plastids	Vidi et al. (2007)
Tobacco	Transformation protocol	Plastid targeting sequences and c*re- loxP*	Plastids	Lutz et al. (2006)
Poplar	*Gfp*	Poplar plastid genes, *accD* and *rbcL*	Plastids	Okumura et al. (2006)
Tobacco	*EBV VCA* antigen	Transplastomic plants	Plastids	Lee et al. (2006b)
Lettuce	*gfp*:: *addA* gene	Plastid genome sequences allowing its targeted insertion between the *rbcL* and *accD* genes	Plastids	Kanamoto et al. (2006)
Rice	*gfp*:: addA	Plastid targeting sequence	Plastids	Lee et al. (2006b)
Petunia	*aadA* :: g*usA* genes	Chloroplast targeting sequence	Plastids	Zubko et al. (2004)
Cotton	*aphA-6* and *npt*II	Plastid transformation vector	Via tobacco chloroplast	Kumar et al. (2004)
Soybean	*aadA*	Plastome sequences targeted between the *trnV* gene and the *rps12/7* operon	Plastids	Dufourmantel et al. (2004)

8.2.2 Mitochondria

The mitochondrial genome is much larger than the plastid genome but considerably smaller than the nuclear genome. The mitochondrial genome size varies from 1,500 to 2,300 kbp in cucumber to about 150 kbp in yeast (Havey et al. 2002). The similarity between plastids and mitochondria raised the possibility that mitochondrial gene may function in plastids and could be included in strategies to transform plastids (Maliga 2004) by designing suitable constructs. Further, Weber-Lotfi et al. (2009) reported that mitochondrial DNA can accept linear DNA molecules. However, the formulation of effective techniques of transforming mitochondria unlike plastids is still underway. There are only a few reports of genetic transformation of mitochondria, for example, in *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii* (Bonnefoy et al. 2007).

8.3 Achievements and Technique Used in Organelle Transformation

8.3.1 Plastids

8.3.1.1 Biolistics Transformation

Development of Transplasteomic Plants Expressing Florescent Genes or Selectable Antibiotic Markers

Davarpanah et al. (2009) transformed plastids of *Nicotina tobacum* following biolistics (standard gene gun) protocol originally adopted by Jeong et al. (2004) by subjecting leaf blades (~3 cm long) previously grown in tissue culture medium with *gfp* and *addA* marker gene (spectinomycin resistance). Similarly, De Marchis et al. (2009) used particle bombardment of sugar beet (*Beta vulgaris*) petioles (of 0.5-cm pieces' size) placed on Petri dishes to obtain transplasteomic plants expressing Gfp (Green Fluorescent Protein). Lee et al. (2006b) obtained transgenic plants expressing *gfp*::*addA* genes thus devising a proper system for rice plastid transformation. Okumura et al. (2006) used biolistic transformation with gold particles (0.6-μm diameter) and 900-psi rupture disk (Bio-Rad Laboratories) to introduce *gfp* genes in poplar. Skarjinskaia et al. (2003) reported plastid transformation of *Lesquerella fendleri* using biolistics for *gfp* and *aadA* transgene. This was the first reported plastid transformable species beyond *Arabidopsis thaliana* of the Brassicaceae family.

Development of Transplasteomic Plants Producing Proteins for Pharmaceutical and Nutraceutical Industries

Scotti et al. (2009) bombarded [Bio-Rad (Hercules, CA, USA) PDS-1000/He] *Nicotiana tabacum* leaves with 0.6-μm diameter gold particles coated with pFA1

or pNS40 plasmid DNA to obtain plants expressing HIV Gag (Pr 55) proteins [a Gag Pr55 protein is a derivative protein from human immunodeficiency virus (HIV), andmyristoyl coenzyme A]. Zhou et al. (2008) obtained transplasteomic tobacco and tomato plants expressing HIV antigen 24 by particle bombardment. Lenzi et al. (2008) obtained transplastomic plants after biolistic transformation using tungsten (1 μm) or gold particles (0.6 μm) as macrocarrier, and DuPont PDS1000He biolistic device. The plasmid was carrying a chimeric gene encoding the L1 protein, in the native viral [*L1(v)* gene] form or in the form of a synthetic sequence suitable for plant plastids [*L1(p)* gene] under the influence of a plastid gene. Lutz et al. (2006) used biolistic transformation to transform plastid genome by homologous recombination. The transgene excision was achieved by plastid targeted *Cre* gene expression from nucleus. Thus, they showed the feasibility of using *Cre-Lox* system in plants. Lee et al. (2006a) showed the feasibility of production of vaccine EBV (Epstein-Barr Virus antigen, which is a source of major cause of malignancies originating from lymphoid and epithelial cells) from transplasteomic tobacco cv. SR1. Moreover, transplastomic tobacco has been cited in several research papers for the production of vaccine antigen against plague, tetanus, anthrax, insulin like growth factors by using biolistic transformation (Daniell et al. 2001; Watson et al. 2004).

Development of Transplasteomic Plants for Crop Improvement

Wurbs et al. (2007) developed a system to facilitate the feasibility of genetically modifying nutritionally important biochemical pathways in nongreen plastids by introducing β-cyclase transgene in the chloroplast genome of tomato. Leelavathi et al. (2003) expressed xylanase enzyme in the chloroplast of tobacco plants. Overproduction of xylanase did not affect plant growth unlike nuclear expressed trans-xylanase enzymes. This provided an example of superior performance of organellar transformation over nuclear transformation. Salt tolerance is another trait, which needs immediate attention. Plant cells tend to accumulate osmoprotectants like glycine betaine to overcome salt-induced stress. Overexpression of such genes in plastids or mitochondria might help a plant to perform well in saline conditions. Kumar et al. (2004) showed cotton plastids can be transformed by plasteomic vectors and this technology can be used for cotton improvement. Dufourmantel et al. (2004) reported the feasibility of transforming plastids of soybean crop, which is an important leguminous and oilseed crop, grown extensively in many parts of the world. There have been reports of development of insect and disease resistance traits, salt- and drought-tolerance traits among important agronomic traits (reviewed by Daniell et al. 2005). Hou et al. (2003) reported chloroplast transformation of oilseed rape and introduction of a specific gene (insect resistance gene *cry1Aa10*) between *rps7* and *ndhB* genes of the plastid genome, thus pioneering a new method for oilseed rape genetic improvement by chloroplast bioengineering.

8.3.1.2 Electroporation

There are only a few reports of using electroporation for transforming plastids in plants. Rangasamy et al. (1997) established plastid targeted transformation of pea using electroporation technique for the expression of ATP:citrate lyase in protoplasts. To et al. (1996) were able to introduce and express *cat* and *gus* genes in isolated spinach plastids by electroporation with pHD203-GUS. Carrot cell cultures have been effectively transformed by electroporation, carrying transplasteomic cells. The rate of growth is very high in culture medium and thus can be used for oral vaccine production programs (Daniell et al. 2005).

8.3.1.3 Other Systems

There are other numerous novel transformation systems, which include microablation (Kajiyama et al. 2008), electrophoresis of embryos, the pollen-tube pathway, microinjection (Neuhaus and Spangenberg 1990; Holm et al. 2000), microbeads' transformation, laser cell perforation (Weber et al. 1988), liposome-mediated gene transfer (Ciboche 1990), and ultrasonification (Joersbo and Brunstedt 1992). These techniques might play an important role in organelle transformation.

8.3.2 Mitochondria

A robust transformation strategy targeting mitochondria instead of the nucleus will allow scientists to use reverse genetics strategy to study trans-mitochondrial gene expression (Havey et al. 2002). This will further establish the efficacy of using target mitochondrial gene to develop transgenic crops. Villarreal et al. (2009) indicated the possibility of overexpressing mitochondria gamma carbon anhydrase-2 gene that causes male sterility in *Arabidopsis*. Possibility of using such genes specifically targeted in mitochondria can help to achieve self-incompatible lines for crop improvement programs. Almost two decades earlier, Kemble et al. (1988) showed the possibility of transforming mitochondria of *Brassica napus* hybrid plants via protoplast fusion mediated by polyethylene glycol (PEG) or electroporation with recombinant vectors. Traits like cytoplasmic male sterility and nonchromosomal stripe mutations of maize have their genomic basis in mitochondria (reviewed by Havey et al. 2002).

8.4 Conclusions

Organelle transformation has opened many vista for crop improvement, production of industrial grade enzymes, biomolecules, pharmaceuticals, and nutraceuticals. More than 40 plastids from different species have been sequenced; thus, a vast plethora of information is available for data mining and development of new

vectors for plastid transformation (Maier and Schmitz-Linneweber 2004). It overcomes the limitations imposed by the nuclear transformation techniques by introducing site-specific genes into plastid genome. Organelle transformation has the advantage as genetic information are maternally inherited against biparental inheritance of the nuclear genome (Daniell 2007). Besides the *cre-lox* system (for preventing transgene escape) can be used as an additional safeguard (Corneille et al. 2003) that can eliminate the risk of transgene escape by pollen release (pollen do not carry plastids or mitochondria). In addition, increasing biosafety of transplasteomic crops (Ruf et al. 2007) should result in more acceptable technology for developing transgenic crops. Organelle transformation is more predictable, can insert many (trans)genes at the same time, is not constrained by gene silencing, and facilitates accumulation of transgene-induced metabolites at high concentration. However, only a limited number of crop species have been exploited for plastid transformation, viz., tobacco (Scotti et al. 2009), petunia (Zubko et al. 2004), tomato (Wurbs et al. 2007) , poplar (Okumura et al. 2006), lettuce, rice, soybean (Dufourmantel et al. 2004), cotton (Kumar et al. 2004). Among them, tobacco has been by far the most successful as compared to other plant species. The flanking sequences used for designing vector for plastid transformation should have 100% homology between plastid genome of different species. Less homology between flanking regions has shown to result in lower transformation efficiency (Zubko et al. 2004). Therefore, emphasis should be paid on species-specific flanking sequences for plastid transformation particularly for field crop species to avoid any complications (Daniell et al. 2005). Thus, the need of the hour is to look at various techniques of transformation as these still remain a bottle neck in the development of transgenic variety using organelle transformation particularly for cultivated crop species.

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Chapter 9
Biosynthesis and Biotransformation

Hajiem Mizukami and Hiroaki Hayashi

9.1 Introduction

Plant secondary metabolites, the so-called natural products, are used as flavors, food additives, and pharmaceuticals. Since complex natural products are not economically produced by total chemical synthesis except for some small molecules, most of the useful metabolites are still obtained from wild or cultivated plant resources. Alternatively, plant cell culture technique would be a potential method to produce these useful secondary metabolites, and commercial production of some useful secondary metabolites has been achieved (Kolewe et al. 2008; Smetanska 2008). Furthermore, biotransformation by plant cell cultures could be a useful method to convert natural products or unnatural synthetic products into chemically different products of economical importance. This chapter will focus on biosynthesis and biotransformation of useful chemical compounds using plant cell culture technology.

9.2 Biosynthesis of Useful Secondary Metabolites by Plant Cell Cultures

Higher plants produce small organic molecules of diverse structures, such as alkaloids, terpenoids, and flavonoids, which are localized in the specific organs of intact plants. Extensive studies showed that some of the useful metabolites are produced by plant cell suspension cultures, while others are not produced by the undifferentiated cells. Some of them are produced by organ culture, especially root cultures and hairy root cultures. However, some useful metabolites are produced

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neither by undifferentiated cells nor by hairy root cultures. In this section, useful secondary metabolites will be discussed under three groups, based on the strategies of production by plant cell cultures: (1) metabolites produced by cell suspension cultures, (2) metabolites produced by hairy root cultures, (3) metabolites that are hardly produced by plant cell/organ cultures. These three classes of metabolites will be discussed using seven selected metabolites as examples in the following subsections, with a focus on the secondary metabolites used for pharmaceuticals.

9.2.1 Useful Secondary Metabolites Produced by Cell Suspension Cultures

Table 9.1 shows some examples of useful secondary metabolites produced by cell suspension cultures. Two outstanding examples of the metabolites are shikonin and paclitaxel, both of which are commercially produced by undifferentiated plant cells. Thus, in this subsection, production of shikonin by *Lithospermum erythrorhizon* cells and paclitaxel by *Taxus* cells will be discussed.

9.2.1.1 Shikonin

Shikonin derivatives, fatty acid esters of shikonin, are red pigments isolated from the roots of *L. erythrorhizon* (Family Boraginaceae) that have been used as a traditional medicine in Japan and China in the form of an ointment to treat wound, burn, and anal hemorrhage (Tabata 1996). Shikonin derivatives have also been used as a dyestuff from ancient times. These red pigments exhibit various pharmacological activities including wound healing, anti-inflammatory, antitumor, and antimicrobial activities (Papageorgiou et al. 1999).

Callus cultures established from seedlings of *L. erythrorhizon* successfully produced shikonin derivatives when the callus was incubated in the dark (Tabata et al. 1974), and repeated cell selection resulted in the establishment of a high shikonin producing culture strain, M18, whose shikonin content was higher than that of the intact roots (Mizukami et al. 1978). Production of shikonin derivatives

Table 9.1 Examples of useful secondary metabolites produced by cell suspension cultures

Metabolite	Plant species	Application	Reference
Shikonin (quinone)	*Lithospermum erythrorhizon*	Dyes, cosmetic	Tabata et al. (1974)
Paclitaxel (diterpenoid)	*Taxus* spp.	Antitumor	Yukimune et al (1996)
Berberine (alkaloid)	*Coptis japonica* *Thalictrum minus*	Antibacterial	Sato and Yamada (1984) Nakagawa et al. (1984)
Sanguinarin (alkaloid)	*Papaver somniferum*	Antibacterial	Eilert et al. (1985)
Ginseonoside (saponin)	*Panax ginseng*	Tonic	Furuya et al. (1983)
Soyasaponin (saponin)	*Glycyrrhiza glabra*	Hepatoprotective	Hayashi et al. (1990)

was completely inhibited by light and a synthetic plant hormone, 2,4-dichlorophenoxyacetic acid (2,4-D). These dramatic regulations of shikonin biosynthesis prompted further experiments to identify many positive-regulating factors such as agar, copper ion, oligogalacturonide, and methyl jasmonate, as well as many negative-regulating factors including light, 2,4-D, ammonium ion, and glutamine (Tabata 1996; Yazaki et al. 1997). Furthermore, establishment of the production medium M9 containing no ammonium ion but nitrate ion for pigment formation (Fujita et al. 1981) led to the commercial production of shikonin by the Mitsui Petrochemical Company (Tabata and Fujita 1985). Thus *L. erythrorhizon* cell culture system provides us with a model system suitable to elucidate the regulatory mechanism of secondary metabolism in higher plants.

Biosynthesis pathway of shikonin and its related compounds has been extensively studied, whereas the subsequent steps of shikonin biosynthesis are still unknown (Tabata 1996). Figure 9.1 depicts the biosynthetic pathways of shikonin and the related constituents in *L. erythrorhizon* cultures. Shikonin is biosynthesized

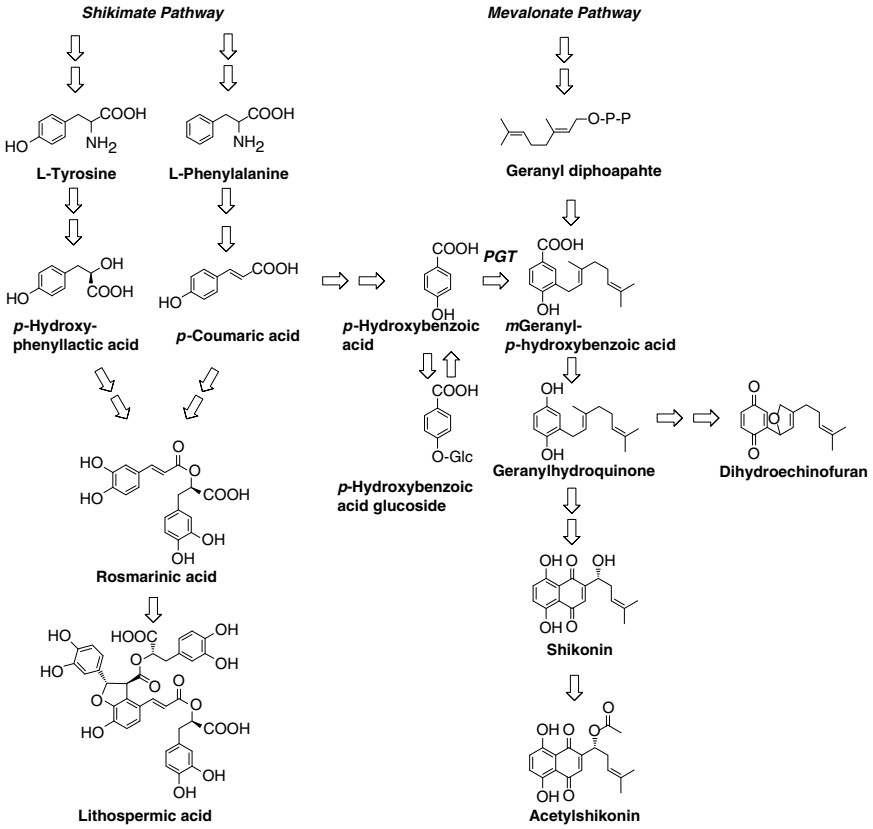

Fig. 9.1 Biosynthetic pathways of acetylshikonin and the related compounds in *Lithospermum erythrorhizon*

from *m*-geranyl-*p*-hydroxybenzoic acid, a coupling product of *p*-hydroxybenzoic acid derived from the shikmate pathway with geranyl diphosphate derived from isoprene units. This coupling step is catalyzed by *p*-hydroxybenzoate geranyltransferase (PGT), which plays a crucial role in the regulation of shikonin biosynthesis (Heide et al. 1989). Two PGT cDNAs have been isolated and characterized from *L. erythrorhizon*, and the regulation of their expression by various physical and biochemical factors coincides with that of shikonin biosynthesis (Yazaki et al. 2002). It is also noteworthy that geranyl moiety of shikonin was shown to be formed via the mevalonate pathway (Li et al. 1998), whereas most of monoterpenes are produced via the MEP pathway, the so-called non-mevalonate pathway (Rohmer 1999).

Not only shikonin derivatives but also dihydroechinofuran, *p*-hydroxybenzoate (PHB) glucoside, and caffeic acid oligomers are accumulated in the cultured *L. erythrorhizon* cells (Tabata 1996; Yamamoto et al. 2002). Production of both shikonin and dihydroechinofuran, an unusual metabolite derived from *m*-geranyl-*p*-hydroxybenzoic acid, is induced by addition of oligogalacturonides or methyl jasmonate to the cultures (Tani et al. 1993; Yazaki et al. 1997), although the accumulation of dihydroechinofuran precedes that of shikonin during the induction by the elicitors. PHB glucoside is regarded as a storage form of PHB, a biosynthetic intermediate of the shikonin biosynthesis, and the accumulation of PHB glucoside is induced by light irradiation, which inhibits the shikonin biosynthesis (Tabata 1996). In addition, the cultured *Lithospermum* cells produce large amounts of caffeic acid oligomers, such as rosmarinic acid, lithospermic acid B, and (+)-rabdosiin derived from phenylpropanoid pathway, whose biosynthesis is regulated differently from that of shikonin derivatives (Yamamoto et al. 2002).

9.2.1.2 Paclitaxel

Paclitaxel isolated from the bark of pacific yew, *Taxus brevifolia* (Family Taxaceae), is an antimitotic drug used in chemotherapy of breast, ovarian, and lung cancers. Since the supply of paclitaxel by isolation from the bark of the slow-growing yew tree is limited, alternative source of paclitaxel was indispensable for its clinical application (Frense 2007). However, the economic production of paclitaxel by total chemical synthesis has not yet been achieved. Alternatively, paclitaxel was produced by semi-synthesis from 10-deacetylbaccatin III, which is a biosynthetic intermediate of paclitaxel and could be obtained from the needle of European yew, *Taxus baccata*, in relatively large amounts. As a more economical source to supply paclitaxel, *Taxus* cell suspension cultures is promising (Frense 2007). Production of paclitaxel by plant cell suspension cultures has been intensively studied, and production of paclitaxel in cell suspension cultures was shown to be significantly up-regulated by methyl jasmonate (Yukimune et al. 1996). Now, paclitaxel is commercially produced by plant cell culture technique using the undifferentiated cells of *Taxus* plants (Frense 2007; Kolewe et al. 2008).

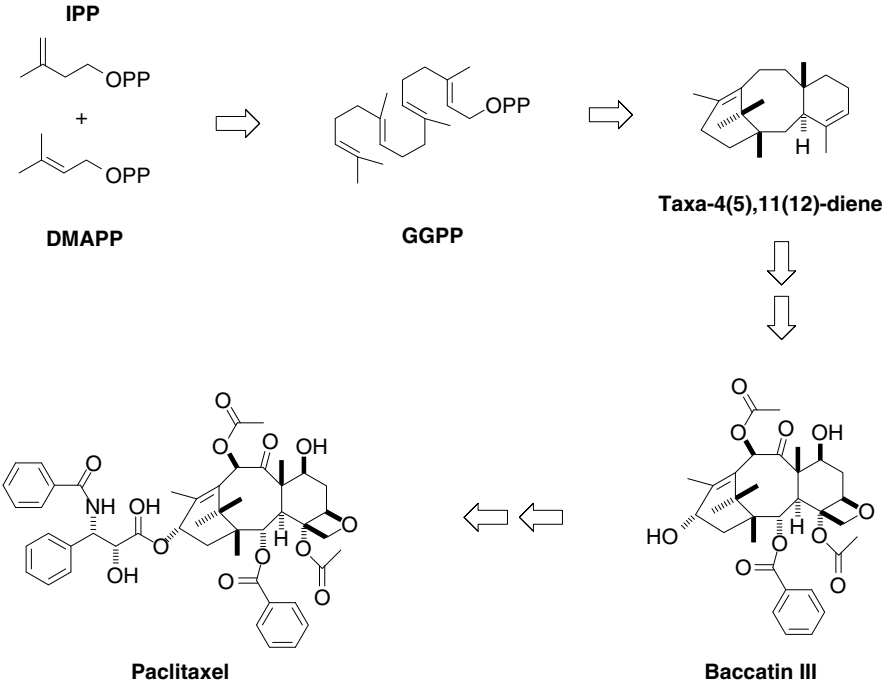

Fig. 9.2 Biosynthetic pathway of paclitaxel in *Taxus* Plants

Since paclitaxel and its analogs are important anticancer drugs, biosynthesis of paclitaxel has been extensively studied to characterize various genes encoding the enzymes involved in the paclitaxel biosynthesis (Jennewein and Croteau 2001; Frense 2007). Figure 9.2 describes the biosynthetic pathway of paclitaxel. Geranylgeranyl diphosphate derived from the MEP pathway was cyclized by a terpene synthase, taxa-4(5),11(12)-diene synthase, into a cyclic diterpene, taxa-4(5),11(12)-diene. The cDNA encoding taxa-4(5),11(12)-diene synthase has been cloned and characterized (Wildung and Croteau 1996). Taxa-4(5),11(12)-diene is further hydroxylated and acylated by several complex steps to produce paclitaxel. Many of genes involved in the later steps have also been characterized (Jennewein and Croteau 2001; Frense 2007).

9.2.1.3 Other Useful Metabolites Produced in the Undifferentiated Cells

Besides shikonin and paclitaxel, many useful secondary metabolites are produced by plant cell suspension cultures as shown in Fig. 9.3. Regarding alkaloids, an antimicrobial isoquinoline alkaloid berberine is produced in the undifferentiated cells of *Coptis japonica* (Sato and Yamada 1984) and *Thalictrum minus* (Nakagawa et al. 1984). Berberine is one of the few secondary metabolites whose biosynthetic

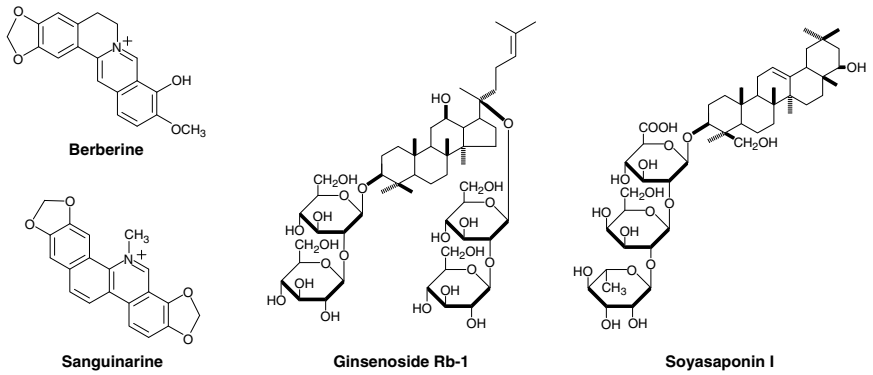

Fig. 9.3 Structures of useful metabolites produced by cell suspension cultures

pathway has been completely characterized (Hashimoto and Yamada 1994). Another antimicrobial alkaloid sanguinarin is produced in the fungal elicitor treated cell suspension cultures of the opium poppy, *Papaver somniferum*, whereas the narcotic alkaloid morphine is not produced in the undifferentiated opium cells (Eilert et al. 1985). Biosynthesis of morphine will be discussed under Sect. 9.2.2.3.

Certain triterpene saponins are also produced by undifferentiated cells of plant cell cultures. Cell suspension cultures of *Panax ginseng* produce ginsenosides, which are active constituents of ginseng, a famous tonic used in the Far East Asia (Furuya et al. 1983). The content and the composition of ginsenosides in cell suspension cultures are almost the same as those in the roots of the cultivated ginseng. Cell suspension cultures of *Glycyrrhiza glabra* (licorice) produce soyasaponins, common triterpene saponins in legumes. However, they do not produce the sweet tritepene saponin, glycyrrhizin, which is localized in the thickened roots of the intact plants (Hayashi et al. 1990). Triterpenoid biosynthesis in licorice will be further discussed under Sect. 9.2.3.2.

9.2.2 Useful Secondary Metabolites Produced by Hairy Root Cultures

Many plant cell suspension cultures have failed to produce useful secondary metabolites that are produced in the respective intact plants. Alternatively, some of these metabolites are produced by organ cultures, such as root cultures and shoot cultures (Kolewe et al. 2008; Smetanska 2008). In particular, hairy root cultures obtained by transforming plant cells with *Agrobacterium rhizogenes* have been used to produce useful secondary metabolites that are not produced in cell suspension cultures (Georgiev et al. 2007). Table 9.2 includes some examples of useful secondary metabolites that are not produced by the undifferentiated cells

Table 9.2 Examples of useful secondary products produced by hairy root cultures

Metabolite	Plant species	Application	Reference
Camptothecin (alkaloid)	*Camptotheca acuminata*	Antitumor	Lorence et al. (2004)
	Ophiorrhiza pumila		Saito et al. (2001)
Hypscyamine (alkaloid)	*Atropa belladonna*	Anticholinergic	Kamada et al. (1986)
	Datura stramonium		Payne et al. (1987)
Scopolamine (alkaloid)	*Scopolia japonica*	Anticholinergic	Mano et al. (1986)
	Duboisia leichhardtii		Mano et al. (1989)
Morphine (alkaloid)	*Papaver somniferum*	Narcotic analgesic	Park and Facchini (2000)
Artemisinin (sesquiterpene)	*Artemisia annua*	Antimalarial	Weathers et al. (1994)
Saikosaponin (saponin)	*Bupleurum falcatum*	Anti-inflammatory	Kim et al. (2006)

but produced by the hairy root cultures. In this subsection, production of tropane alkaloids and camptothecin will be discussed.

9.2.2.1 Tropane Alkaloids

Tropane alkaloids, hyoscyamine and scopolamine, are isolated from certain Solanaceaus plants including *Atropa belladonna, Datura innoxia, Duboisia leichhardtii, Hyoscyamus niger*, and *Scopolia japonica*. They are muscarinic antagonists used for treatment of various gastrointestinal disorders. Callus cultures of *Scopolia parviflora* produce only trace amounts of tropane alkaloids, whereas the root-differentiated tissue accumulates higher content of tropane alkaloids (Tabata et al. 1972). Similar results were observed in other tropane alkaloid producing plants, such as *A. belladonna*, *Datura stramonium*, and *D. leichhardtii*. In contrast, undifferentiated cell suspension cultures of *H. niger* produced small amount of tropane alkaloids (Yamada and Hashimoto 1982; Yamada and Endo 1984). In the intact plants, tropane alkaloids are produced mainly in the root and are translocated into the aerial parts, which have the storage organs of these alkaloids (Waller and Nowacki 1978). Thus, hairy root cultures of these tropane alkaloid producing plants were established to produce alkaloids. As expected, hairy root cultures of *S. japonica* (Mano et al. 1986), *A. belladonna* (Kamada et al. 1986), *Datura stramonium* (Payne et al. 1987), and *D. leichhardtii* (Mano et al. 1989) were shown to produce high levels of tropane alkaloids.

Biosynthetic pathway of tropane alkaloids is extensively studied using the root cultures as shown in Fig. 9.4 (Hashimoto and Yamada 1994). Tropane alkaloids are esters of tropine and tropic acid, which are derived from arginine and phenylalanine, respectively. The most characterized enzyme in the biosynthetic pathway of tropane alkaloids is hyoscyamine-6-hydroxylase (H6H), a 2-oxoglutarate-dependent dioxygenase, involved in the conversion of hyoscyamine into scopolamine. This enzyme is localized in the pericycle of the root (Hashimoto et al. 1991). This

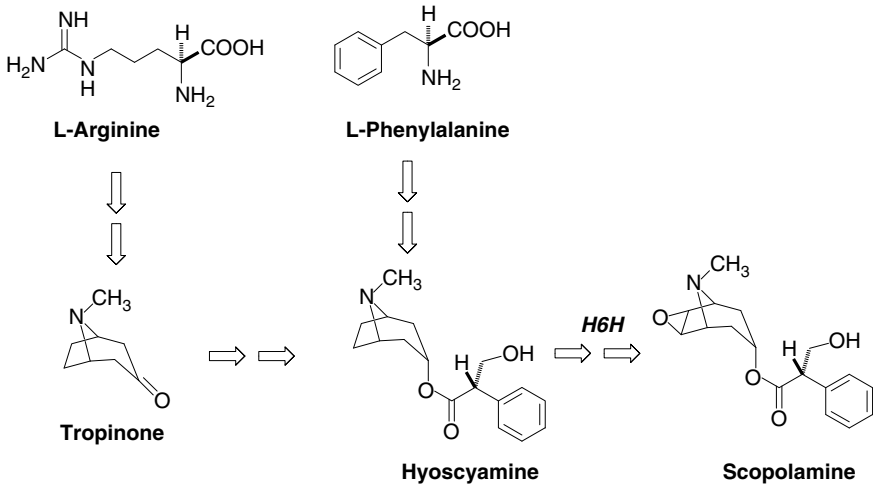

Fig. 9.4 Biosynthetic pathway of tropane alkaloids

enzyme is involved not only in the hydroxylation of hyoscyamine to 6-hydroxyhyoscyamine but also in the epoxidation of 6-hydroxyhyoscyamine to scopolamine. H6H cDNA has been isolated from *H. niger* (Matsuda et al. 1991), and metabolic engineering using the H6H gene resulted in transgenic *A. belladonna* with the preferential accumulation of scopolamine, which has a higher commercial demand than hyoscyamine (Yun et al. 1992).

9.2.2.2 Camptothecin

Camptothecin is an anticancer compound isolated from *Camptotheca acuminata* (Family Nyssaceae). Although camptothecin itself is not used for cancer chemotherapy due to its toxicity, the semisynthetic compounds derived from camptothecin, such as irinotecan and topotecan, are clinically important antitumor drugs in the world (Fig. 9.5). Since economical total synthesis of camptothecin has not been achieved, plant cell cultures can be an alternative method to produce camptothecin (Lorence and Nessler 2004).

Cell suspension cultures of *C. acuminata* were established, but they produced only trace amounts of camptothecin (Sakato et al. 1974). Later, hairy root cultures of *C. acuminata* were found to produce significant amount of camptothecin (Lorence et al. 2004). Camptothecin is produced not only by *C. acuminata* but also by many other species of taxonomically unrelated families including *Nothapodytes foetida* (Family Icacinaceae) and *Ophiorrhiza pumila* (Family Rubiaceae) (Lorence and Nessler 2004). Although undifferentiated callus cultures of *O. pumila* produced no camptothecin (Kitajima et al. 1998), hairy root cultures of *O. pumila* produced high levels of camptothecin (Saito et al 2001). Furthermore, a part of camptothecin produced by *O. pumila* hairy roots was excreted into culture media, and the excreted

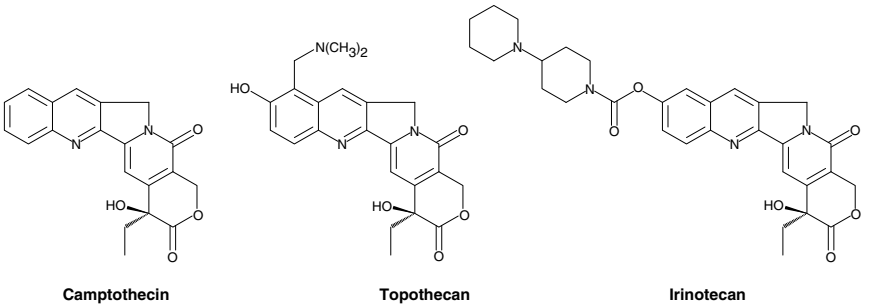

Fig. 9.5 Camptothecin and anticancer drugs (topothecan and irinotecan) derived from camptothecin

camptothecin in the medium was efficiently recovered by resin. This system is feasible to supply camptothecin from plant cell cultures.

Camptothecin is a monoterpene indole alkaloid biosynthesized from a key intermediate strictosidine. Strictosidine is the condensation product of tryptamine, a monoamine alkaloid derived from shikimate pathway, with secologanin, an iridiod glycoside (for the biosynthetic pathway see Fig. 9.8). It has been shown that the secologanin moiety in the structure of comptothecin is derived from monoterpene formed via MEP pathway (Yamazaki et al. 2004). cDNAs encoding tryptamine decarboxylase and strictosidine synthase, two key enzymes in the monoterpene indole alkaloid biosynthesis, have already been isolated and characterized (Yamazaki et al. 2003). However, later steps of camptothecin biosynthesis are so far unknown.

9.2.2.3 Morphine

Morphine, an important narcotic analgesic, is a benzylisoquinoline alkaloid produced by opium poppy, *Papaver sominiferum* (Family Papaveraceae), which is one of the most thoroughly investigated model plants to elucidate the regulation of alkaloid biosynthesis in higher plants (Facchini and De Luca 2008). Although cell suspension cultures of *P. somniferum* produce no detectable amount of morphine and codeine, the undifferentiated cells treated with fungal elicitors accumulate sanguinarine, another antimicrobial alkaloid distributed in some plants of the family Papaveraceae (Eilert et al. 1985). In contrast, hairy root culture of *P. somniferum* was shown to produce small amount of morphine (Park and Facchini 2000).

Biosynthetic pathways of morphine and sanguinarine have been almost completely characterized (Facchini et al. 2007). Figure 9.6 illustrates the biosynthetic pathways of morphine and related alkaloids. These benzylisoquinoline alkaloids share the central intermediate (S)-norcoclaurine, which is produced by norcoclaurine synthase from dopamine and 4-hydroxyphenylacetaldehyde. (S)-Norcoclaurine is converted into the branching intermediate (*S*)-reticuline. The first committed step in the morphine biosynthesis involves the conversion of (*S*)-reticuline into its (R)-epimer. On the other hand, the conversion of (*S*)-reticuline to

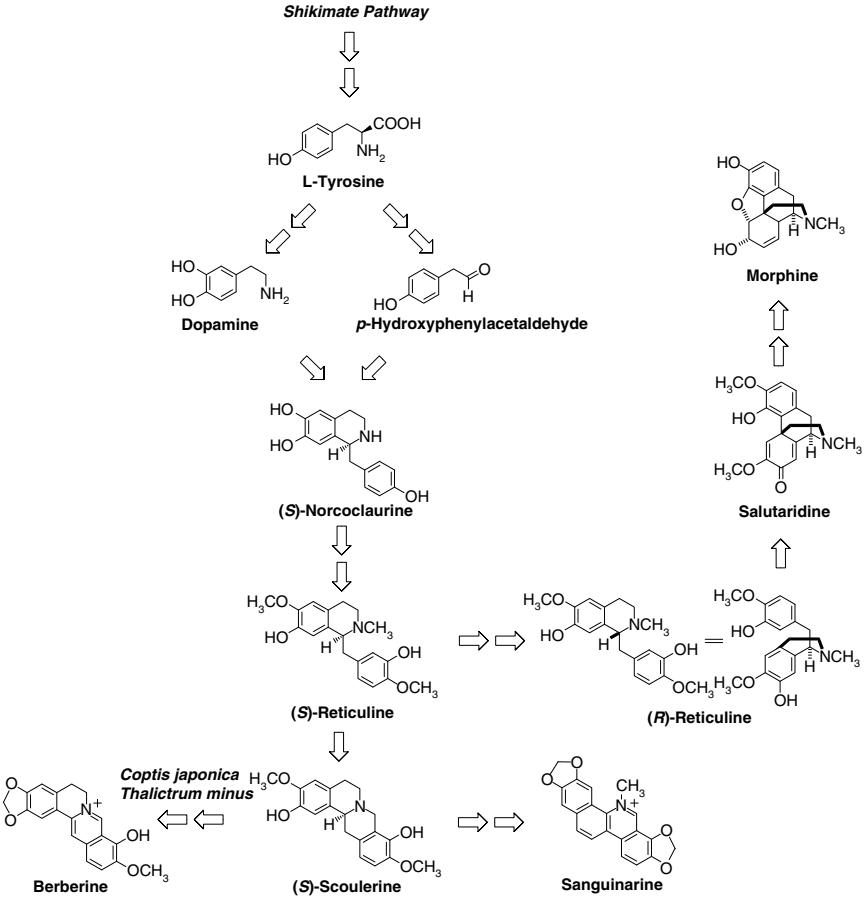

Fig. 9.6 Biosynthetic pathway of morphine and related alkaloids in *Papaver somniferum*

(S)-scoulerine by berberine bridge enzyme leads to sanguinarine in *P. somniferum* as well as to berberine in *C. japonica* (Hashimoto and Yamada 1994; Facchini et al. 2007). Many genes involved in the alkaloid biosynthesis have been so far identified from opium poppy. Extensive studies including the biosynthesis pathway, gene regulation, and metabolic engineering of morphine and related alkaloids are reviewed elsewhere (Facchini et al. 2007; Facchini and De Luca 2008).

9.2.2.4 Other Useful Metabolites Produced by Hairy Root Cultures

In addition to the metabolites mentioned earlier, many other useful secondary products are produced by hairy root cultures, whereas they are not produced by cell suspension cultures. Saikosaponins (Fig. 9.7) are oleanane-type triterpene saponins with antiallergic and anti-inflammatory activities, and are isolated from

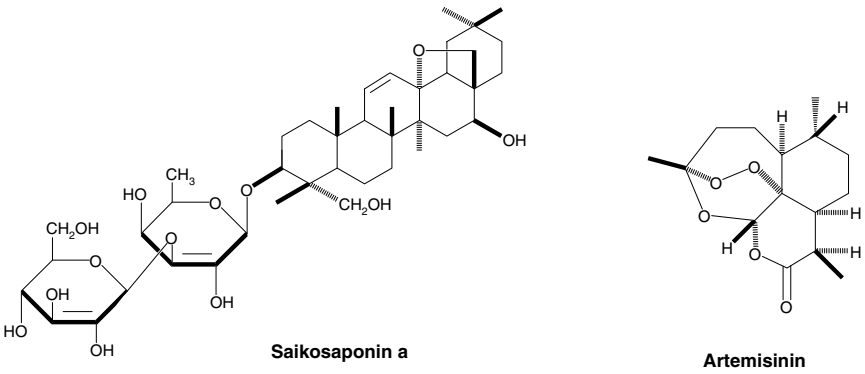

Fig. 9.7 Structures of useful metabolites produced by hairy root cultures

the root of *Bupleurum falcatum*. Although callus cultures of *B. falcatum* produce no detectable amounts of saikosaponins, adventitious roots differentiated from the callus (Uomori et al. 1974) as well as hairy root cultures (Kim et al. 2006) of *B. falcatum* are capable of producing large amount of saikosaponins. Artemisinin (Fig. 9.7), an endoperoxide sesquiterpene lactone isolated from *Artemisia annua*, has a potent antimalarial activity against the chloroquin-resistant malarial parasite *Plasmodium falciparum*; thus, this compound is one of the targets for plant cell cultures (Liu et al. 2006). No artemisinin was produced in the cell suspension cultures of *A. annua,* whereas only trace amount of artemisinin was found in multiple shoot cultures (Paniego and Giuletti 1994). Artemisinin was produced by hairy root cultures of *A. annua*, suggesting that the commercial production of artemisinin by hairy root culture is feasible (Weathers et al. 1994).

9.2.3 Useful Secondary Products That Are Hardly Produced by Plant Cell Cultures

Despite the extensive efforts to produce useful secondary products by the plant cell cultures, some target metabolites are hardly produced by the cell suspension cultures as well as by the hairy root cultures. Examples of these useful products are listed in Table 9.3. In this subsection, dimeric monoterpene indole alkaloids, vincristine and vinblastine in *Catharanthus roseus* and glycyrhrizin in *G. glabra*, are described.

9.2.3.1 Vinca Alkaloids

Madagascar periwinkle, *C. roseus* (syn. Vinca; Family Apocynaceae), produces the Vinca alkaloids vincristine and vinblastine, which are mitotic inhibitors used for clinical treatment of cancer. Both vincristine and vinblastine are dimeric

Table 9.3 Examples of useful secondary metabolites that are hardly produced by plant cell cultures

Metabolite	Plant species	Application	Culture method	Reference
Vinblastine (alkaloid)	*Catharanthus roseus*	Antitumor	Cell suspension	Eilert et al. (1987)
	Hairy root			Parr et al. (1988)
Glycyrrhizin (saponin)	*Glycyrrhiza glabra*	Sweetener	Cell suspension	Hayashi et al. (1988)
	Hairy root			Toivonen and Rosenqvit (1995)

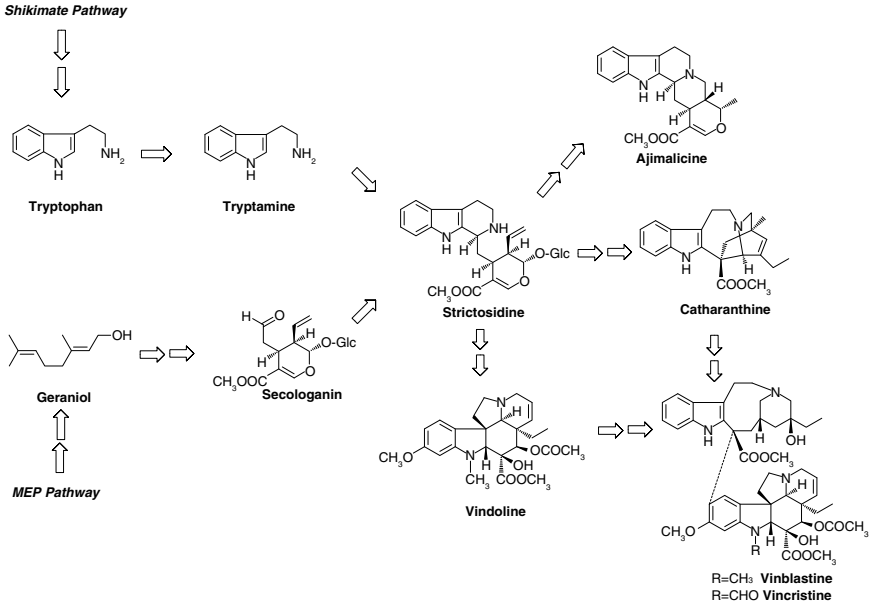

Fig. 9.8 Biosynthetic pathways of vinblastine and vincristine in *Catharanthus roseus*

monoterpene indole alkaloids with complex chemical structures (Facchini and De Luca 2008). The content of Vinca alkaloids is low in the field-grown plants, which are the actual sources to supply these alkaloids at the present. Thus, an alternative method to produce these alkaloids is necessary, and plant cell culture technique would be an attractive method. However, despite the enormous efforts, the dimeric alkaloids are not produced sufficiently by cell suspension culture (Eilert et al. 1987) or by hairy root cultures of *C. roseus* (Parr et al. 1988; Toivonen et al. 1989). To overcome this difficulty, extensive studies including chemistry, biochemistry, and molecular biology have been carried out. In fact, *C. roseus* is one of the most thoroughly investigated medicinal plants. This subject has been well reviewed elsewhere (Facchini and De Luca 2008).

Figure 9.8 depicts the biosynthetic pathways of Vinca alkaloids. Monoterpene indole alkaloids are biosynthesized from a key intermediate, strictosidine, which is the condensation product of tryptamine, a monoamine derived from shikimate pathway, and secologanin, an iridiod glycoside derived from nonmevalonate pathway. Although hairy root cultures of *C. roseus* produce only trace amounts of dimeric monoterpene indole alkaloids, they produce monomeric alkaloids, such as ajmalicine and catharanthine. However, the hairy roots produce no detectable amount of vindoline, which is one of the building blocks of the dimeric indole alkaloids (Toivonen et al. 1989).

9.2.3.2 Glycyrrhizin

Glycyrrhizin is a sweet oleanane type triterpene saponin isolated from the roots and stolons of *G. glabra* (licorice) of the family Fabaceae. Dried roots and stolons of licorice have been used as an important crude drug from ancient times (Shibata 2000). Glycyrrhizin is also used as an anti-inflammatory drug for treatment of hepatitis. Cell suspension cultures of *G. glabra* produced no detectable amount of glycyrrhizin (Hayashi et al. 1988). Glycyrrhizin also was not produced by hairy root cultures of *G. glabra* (Toivonen and Rosenqvit 1995). In the intact plant of *G. glabra,* the accumulation of glycyrrhizin is localized in the thickened roots and stolons, and glycyrrhizin is not contained in the rootlets, leaves, stems, and seeds (Hayashi et al. 1993). Instead of glycyrrhizin, cultured licorice cells produced two triterpenoid constituents, viz. soyasaponins and betulinic acid. Soyasaponins are also oleanane-type triterpene saponin, and are localized mainly in the seed and rootlet of *G. glabra*. Betulinic acid, a lupane-type triterpene distributed widely in higher plants, is mainly localized in the cork layer of the thickened licorice roots.

Figure 9.9 shows the biosynthetic pathways of glycyrrhizin and the related triterpenoids. These triterpenoids and sterols share a common key intermediate, 2,3-oxidosqualene, which is formed via the mevalonate pathway (Rohmer 1999). 2,3-Oxidosqualene is further converted by three oxidosqualene cyclases, β-amyrin synthase (bAS), lupeol synthase (LUS), and cycloartenol synthase (CAS) into the three cyclization products, respectively, leading to the end-products. cDNAs of these three oxidosqualene cyclases are characterized from *G. glabra,* and mRNA levels of the oxidosqualene cyclases were differently regulated in the intact plants and cultured cells of *G. glabra*. The levels of their mRNAs correlate with the accumulation of respective end products indicating that the transcription of oxidosqualene cyclase genes is an important regulatory step for triterpenoid biosynthesis (Hayashi et al. 2003, 2004). The following steps of the saponin biosynthesis pathway are oxidations and glycosylations of triterpenes. Recently, β-amyrin 11-oxidase, a cytochrome P450 involved in glycyrrhizin biosynthesis, has been characterized (Seki et al. 2008). Metabolic engineering of a saponin-producing plant or microorganism is an attractive approach to produce unique and useful saponins in the future.

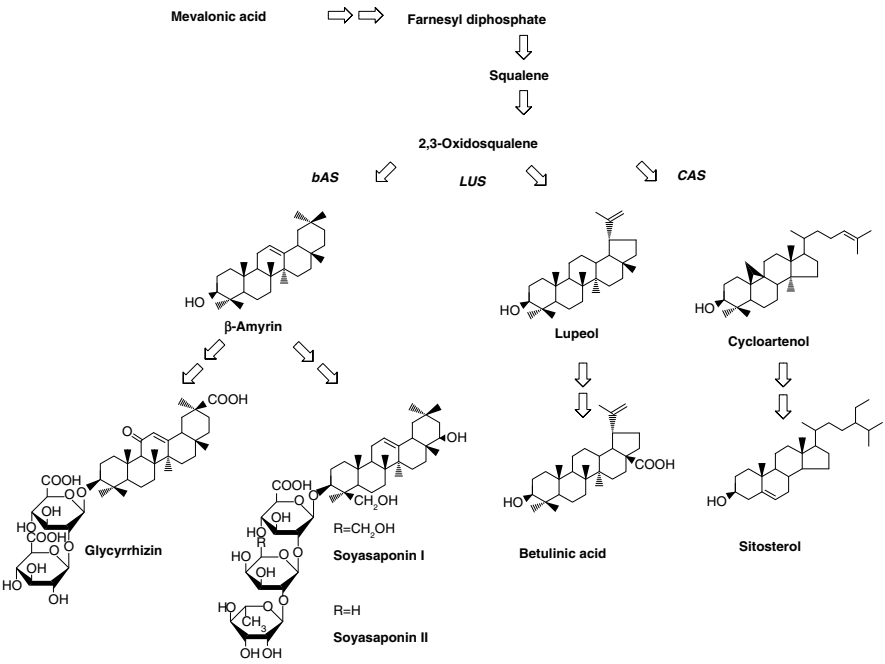

Fig. 9.9 Biosynthetic pathways of glycyrrhizin and related triterpenoids in *Glycyrrhiza glabra*

9.3 Biotransformation

In addition to synthesizing secondary metabolites de novo from carbon sources supplemented in the culture medium, plant cells are able to carry out biotransformation reactions on substrates exogenously added to the culture medium. Not only secondary metabolites but also xenobiotic compounds can be used as substrates of biotransformation. Such biotransformation reactions have been investigated as one of the major targets for biotechnological application of plant cell culture systems because plant cells can catalyze the stereospecific- and/or region-specific modification of organic compounds that are not easily carried out by chemical synthesis or by microorganisms (Rao and Ravishankar 1999). Biotransformation reactions include oxido-reduction, hydroxylation, glycosyl conjugation, acylation, methylation, etc. (Suga and Hirata 1990). This chapter describes biotransformations catalyzed by cultured plant cells, focusing on examples with potential importance for industrial application.

9.3.1 Glycosylation

Higher plants synthesize a wide range of glycosides as secondary metabolites, and are capable of conjugating sugar residues not only to endogenous metabolic

intermediates but also xenobiotics. Glycosyl conjugation of lipophilic low molecular weight compounds is an efficient tool to enhance water solubility, to improve stability, and thereby to increase bioavailability and to modify biological activity. Chemical synthesis of glycosides is usually difficult because it involves multiple blocking/deblocking steps before any product can be obtained. Thus, glycosylation of various organic compounds has attracted attention as one of the targets in biotechnological application of plant cell culture systems. Here we describe some examples of practical interests.

9.3.1.1 Arbutin

Arbutin is a monoglucoside of hydroquinone and a main bioactive compound contained in *Arctostaphylos uva-ursi* that has been traditionally used as a urethral disinfect. Arbutin also inhibits the melanin formation in human (Akiu et al. 1988) and is used as an ingredient of cosmetics. Glucosylation of hydroquinone to arbutin was first shown using *Datura innoxia* cell suspension cultures (Tabata et al. 1976). Later, it was shown that *C. roseus* cells in culture can efficiently convert hydroquinone to arbutin, particularly when medium concentration of sucrose is increased (Yokoyama et al. 1990). A large-scale production system up to 20-L jar fermenter was established by culturing *C. roseus* cells at high density followed by continuous supply of hydroquinone to the medium (Fig. 9.10). The arbutin yield could be increased as high as 9.2 g/L (corresponding to 45% of cell dry weight) and the conversion rate from hydroquinone was 98% (Inomata et al. 1991).

9.3.1.2 Curcumin

Curcumin (diferuloylmethane) is a yellow pigment of turmeric (dried rhizome of *Curcuma longa*). It has been primarily used as a food colorant but has attracted increased attention because of its potent pharmacological activities (Maheshwari et al. 2006). However, its low water solubility limits further pharmacological exploration and practical application. Screening of cell cultures from ten different

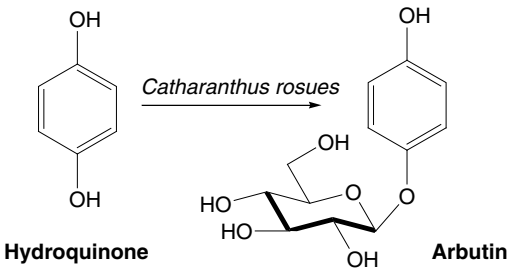

Fig. 9.10 Glucosylation of hydroquinone in *Catharanthus roseus* cell suspension cultures

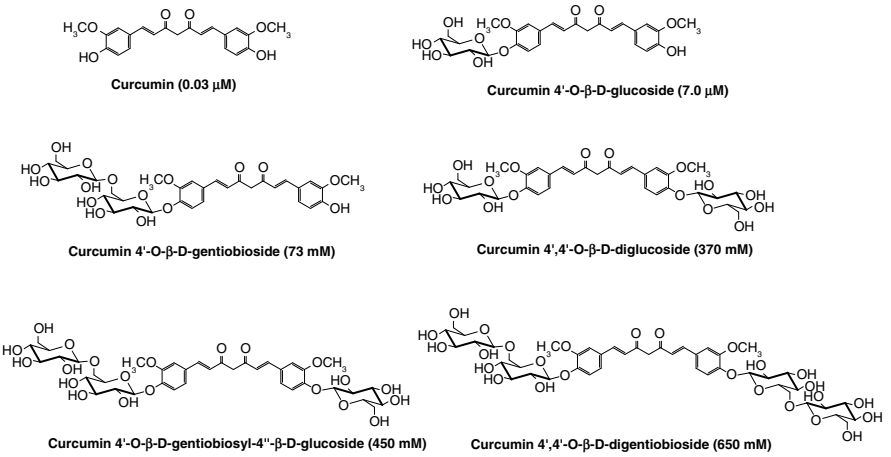

Fig. 9.11 Curcumin glucosides produced from curcumin by *Catharanthus roseus* cell suspension cultures Water solubility of the each compound is shown in a parenthesis

plant species revealed that *C. roseus* cells converted exogenously supplied curcumin to a series of curcumin glucosides as shown in Fig. 9.11 (Kaminaga et al. 2003). Although the water solubility of curcumin monoglucoside was increased by only 230-fold, the solubility was increased about two million-fold and about 20 million-fold in the case of curcumin monogentiobioside and curcumin digentiobioside, respectively, compared to the solubility of curcumin. The result indicated that conjugation of at least two glucose residues to curcumin enhanced dramatically the water solubility. An effective chemoenzymatic system for glucosylation of curcumin was established using a recombinant glucosyltransferase from *C. roseus* (Masada et al. 2007). However, high cost of UDP-glucose, a donor substrate for the enzymatic glucosylation, still makes the biotransformation using cultured cells a method of choice although the product yield should be improved.

9.3.1.3 Capsaicin

Capsaicin is a major pungent compound in hot red peppers (fruits of *Capsicum annum*). It exhibits analgesic activity through stimulation of vanilloid 1 receptor and has been used as a topical analgesic as well as a therapeutic drug against allergic rhinitis (Bley 2004). However, incomplete solubility of capsaicin sometimes leads to variable results of capsaicin activity (Kopec et al. 2008) and limits pharmacological exploitation. Capsaicin was first shown to be glucosylated to the monoglucoside by *Coffea arabica* cultured cells (Kometani et al. 1993). Recently, *C. roseus* suspension cultures was shown to convert capsaicin and 8-nordihydrocapsaicin to their monoglucosides (main products) together with β-primeveroside

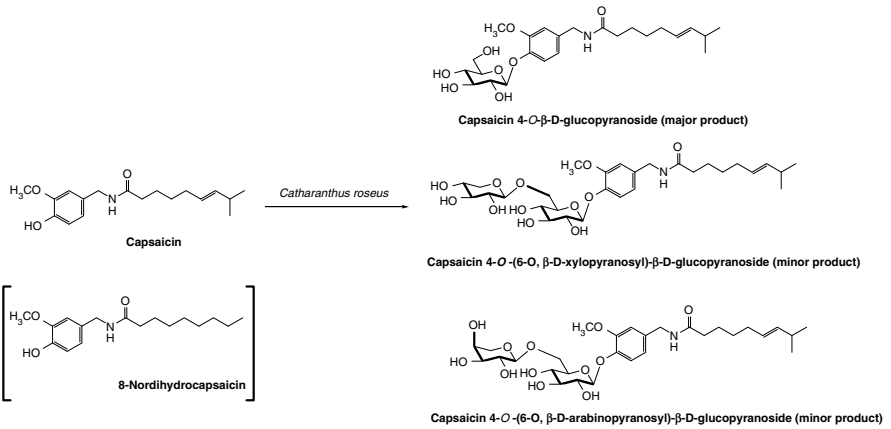

Fig. 9.12 Glycosylation products of capsaicin by *Catharanthus roseus* cell suspension cultures

(xylopyronosylglucopyranoside) and β-vicianoside (arabinopyranosylglucopyranoside), as shown in Fig. 9.12. The glycosylation rate was slightly higher for 8-nordihydrocapsaicin than for capsaicin (Shimoda et al. 2007). Since capsaicin monoglucoside was reported to be 1/100 times pungent as compared to capsaicin (Mihara et al. 1992), while exhibiting higher activity for reducing serum and liver lipid levels in vivo (Tani et al. 2003), it may be interesting to clarify the physiochemical and pharmacological characteristics of these capsaicinoid glycosides.

9.3.1.4 Monoterpene Alcohols

Higher plants produce a variety of flavors. These compounds are likely to sublime and have usually low solubility in water. Glucosylation of these volatile compounds has been one of the targets for biotransformation in plant cell cultures.

Menthol is a monoterpene compound contained in the essential oils from *Mentha* plants and has been used as a refreshing flavor for foods, medicines, and cosmetics. However, practical use of menthol is limited because of its low water solubility. *Eucalyptus perririana* cells glucosylated either (+)- or (−)-menthol mainly to their gentiobisosides (Orihara et al. 1991). Although glucosylation yield was much lower for (+)-menthol than that for (−)-menthol, a triglucoside, 2,6-di-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside, was produced only from (+)-menthol as a minor product. Monoterpene alcohols such as thymol, carvacrol, and eugenol were also efficiently converted to their genitiobiosides presumably via the corresponding monoglucosides by *E. perriniana* cell suspension cultures, and the conversion rate reached as high as about 90% in case of thymol (Shimoda et al. 2006), as shown in Fig. 9.13.

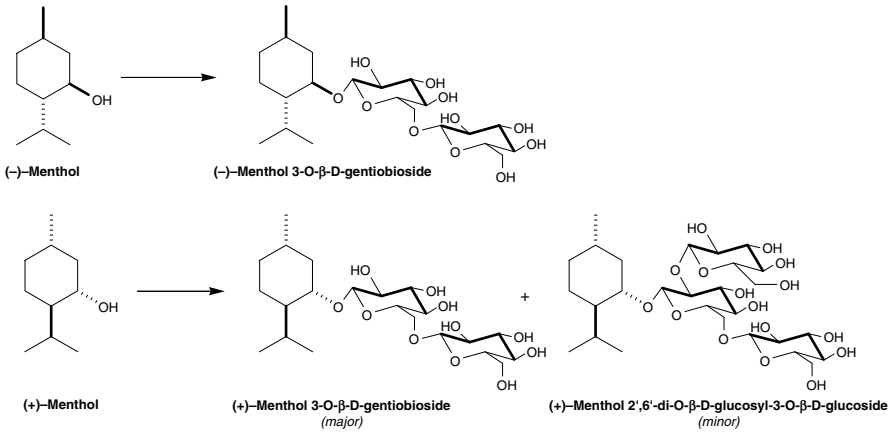

Fig. 9.13 Glycosylation products of (+)-menthol and (-)-menthol by *Eucalyptus perririana* cell suspension cultures

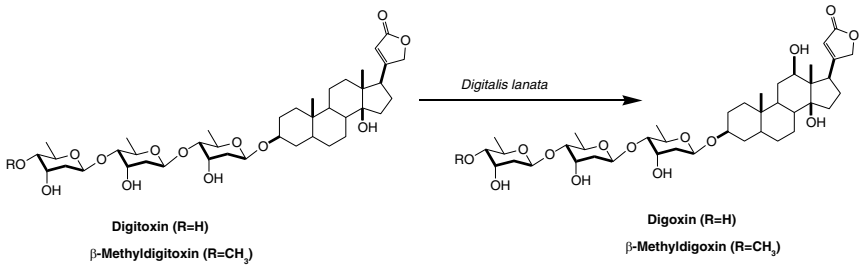

Fig. 9.14 Hydroxylation of digitoxin and β-methyldigitoxin by *Digitalis lanata* cell suspension cultures

9.3.2 Hydroxylation

Regio- and stereo-selective introduction of oxygenated functions at various positions of exogenously supplied molecules by plant cell cultures has been widely investigated because this may lead to modification in biochemical and pharmacological activities of the particular compound.

9.3.2.1 Digitoxin

Digitoxin and digoxin (12β-hydroxydigitoxin) are cardenolides accumulated in *Digitalis purpurea* and *D. lanata*. Although both compounds have been used for treatment of chronic heart diseases, digoxin has superior pharmacological and pharmacodynamic properties compared to digitoxin. In contrast, the amount of

each compound recovered is by far in excess in favor of digitoxin. This led to the investigation of regio- and stereo-specific hydroxylation of digitoxin to produce digoxin by plant cell cultures (Alfermann et al. 1980). *D. lanata* cells were found to hydroxylate digitoxin at 12-position to produce 12β-hydroxydigitoxin (digoxin) (Fig. 9.14). β-Methyldigitoxin was a more suitable substrate than digitoxin because the former compound was efficiently converted to β-methyldigoxin with few by-products. Methyldigoxin yield reached 800 mg/L when *D. lanata* cells were cultured in a 20-L stirred tank reactor (Spieler et al. 1985).

The biocatalytic ability of 12β-hydroxylation seems to be specific to *D. lanata* cells because digitoxingenin was hydroxylated at 1β- or 5β-position by *Strophanthus gratus*, *S. intermedius,* and *Daucus carota* cells (Furuya et al. 1988; Kawaguchi et al. 1989) and at 16β-position by *D. purpurea* cells (Hirotani and Furuya 1980). It may be interesting to investigate the pharmacological activities of these hydroxylated digitoxins at 1β-, 5β-, or 16β-position because they have not been found in nature.

9.3.3 Miscellaneous

9.3.3.1 Podophyllotoxin

Podophyllotoxin in an antineoplasmic lignan isolated from *Podophyllum peltatum*. Etoposide is an important anticancer drug chemically derived from podophyllotoxin and clinically used for treatment of small cell lung carcinoma. Etoposide is synthesized through chemical conversion of podophylltoxin to 4′-demethylepipodophyllotoxin, which is then attached with the carbohydrate unit leading to etoposide. Because the present route requires isolation of podophyllotoxin from *P. peltatum*, an alternative route to 4′-demethylepipodophyllotoxin was exploited using synthetic dibenzylbutanolide as a substrate. *P. peltatum* cells in culture converted exogenously added dibenzylbutanolide to a podophyllotoxin analog with a 50% conversion rate (Kutney et al. 1993). Later, it was found that the cyclization of dibenzylbutanolide was performed with peroxidase excreted into culture broth from cells of different species such as *Nicotiana sylvestris* (Botta et al. 2001) and shoot cultures of *Halplophyllum patavinum* (Puricelli et al. 2003). Although a chemical process in removing the isopropyl group in the biotransformation product and regeneration of the methylenedioxy function was completed, the final product is still a C1-isomer of 4′-demethylepipodophyllotoxin (Fig. 9.15).

9.3.3.2 Scopolamine

Hyoscyamine-6-hydroxylase (H6H) catalyzes conversion of hyoscyamine to its epoxide scopolamine which has higher pharmacological values. Transgenic tobacco cell cultures expressing an H6H transgene derived from *Hyoscyamus*

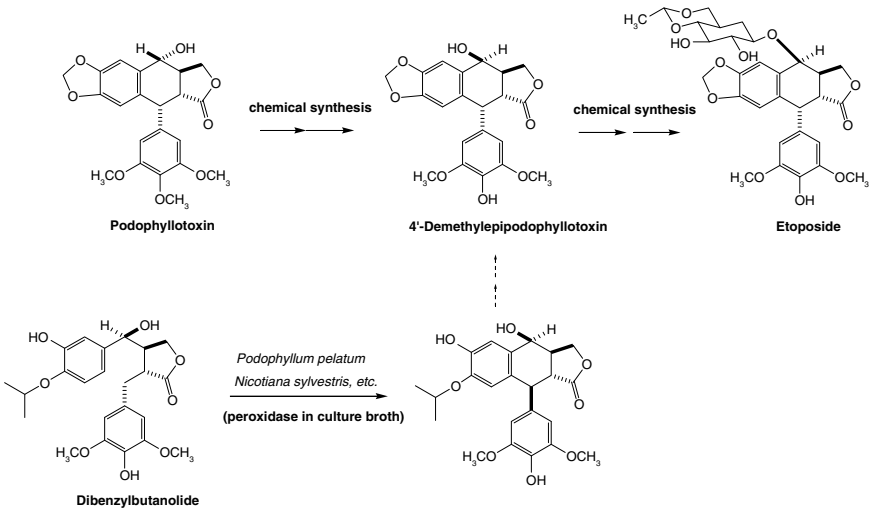

Fig. 9.15 Biotransformation of a dibenzylbutanolide to a podophyllotoxin analog by plant cell cultures

muticus efficiently converted exogenously supplied hyoscyamine to scopolamine. The productivity of scopolamine reached to 36 mg/L when 200 mg/L hyoscyamine was added to the cells cultured in a 5-L turbine stirred bioreactor, corresponding to 18% conversion (Moyano et al. 2007).

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Chapter 10
Metabolic Engineering of Pathways and Gene Discovery

Miloslav Juříček, Chandrakanth Emani, Sunee Kertbundit, and Timothy C. Hall

10.1 Introduction

Humans have been manipulating the genetic information of plants throughout the history of agriculture. In this respect, every new plant variety or animal race is a result of the introduction of novel metabolic changes. This process has been slowly advancing for millennia. However, with the discovery of biochemical pathways and later with the introduction of gene manipulation techniques in 1970s, the pace greatly speeded up. Already in the mid-1980s, many of the compounds and enzymes participating in metabolic pathways were linked to their cloned genes, which can then be used for engineering the plant metabolism. Soon, novel products from plants appeared including, vaccines and other pharmaceuticals, plastics, and proteins that may render certain plants as effective tools for environmental decontamination. These products were a result of the manipulation of plant endogenous biochemical pathways and thus the novel field of science-metabolic engineering was born. Metabolic engineering can be defined as the targeted and purposeful modification of metabolic pathways in an organism for the improved use of cellular pathways for chemical transformation, energy transduction, and macromolecular synthesis or breakdown, potentially benefiting the society by producing biological substitutes for toxic chemicals, increasing agricultural production, improving industrial fermentation processes, producing completely new compounds, or by understanding the molecular mechanism underlying medical conditions in order to develop new cures (Kurnaz 2005).

Among several organisms, plants have rapidly become the main object of metabolic engineering. This may be attributed to the higher interest in plants over

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bacteria or other organisms considered which was stimulated by potential commercial applications resulting from engineering the resistance against pests and diseases and later also improving the contents of metabolites already used in medicine or developing novel medicines. Engineering of secondary metabolites seemed the easiest way to obtain this goal. However, more than hundred thousand metabolites have already been identified and this may be only a fraction of the total amount in plant kingdom. Clearly, detailed mapping of metabolic pathways and their engineering will be an enormous task. Moreover, even detailed understanding of the biochemical processes may not be sufficient because an interaction between various pathways in the total metabolic network, enzyme complexes, compartmentation, feedback inhibition, and/or gene expression regulation may completely change the story. Despite these obstacles, a number of successful cases were reported during the last 30 years, some of them are summarized later.

10.2 The Beginnings and Early Years of Metabolic Engineering

Historical archives of plant sciences related to exploiting plants as natural chemical factories are replete with examples of utilizing plants as sources of medicinal compounds. One of the earliest examples is the medicinally valuable St. John's wort discovered by the Greek physician, Hippocrates, in the fifth century BC. In the present times, St. John's wort is part of the medical research and clinical trials aimed at determining its efficacy for a wide variety of ailments such as depression, cancer, inflammation, and viral infections. The ancient Indian medical discipline of *Ayurveda* effectively illustrates the use of plants as derivatives of medicines in combating various ailments. In modern medicine, one-quarter of prescription drugs are of plant origin (Fischer and Emans 2000).

The technology of extracting useful compounds from plants evolved into what we now know as "plant molecular pharming." In 1983, Murai et al. (1983) demonstrated that a part of bean phaseolin seed protein gene was transcribed in sunflower cells transformed by the tumor-inducing plant vector *Agrobacterium tumefaciens*, the first unequivocal demonstration of the transfer of a developmentally regulated plant gene from one plant species to another. In a similar development, bacterial genes were expressed in higher plants (Fraley et al. 1983), followed by the novel leaf disk *Agrobacterium*-mediated transformation method of Horsch et al. (1985) that combined gene transfer, plant regeneration, and an effective kanamycin-based selection to generate transgenic petunia, tobacco, and tomato. The most commonly used reporter gene to monitor transgene expression, the β-glucuronidase gene (*gusA* or *uidA*) (Jefferson et al. 1987), is one of the earliest examples of a successful molecular pharming product as its commercial production served as a model system for the production of proteins in transgenic corn plants (Kusnadi et al. 1998; Witcher et al. 1998). Prior to this, During (1988) demonstrated the wound-inducible expression and secretion of the T4 lysozyme and monoclonal antibodies in tobacco, the first report of human antibody expression in a transgenic plant. This was

followed by the expression of secretory antibodies in transgenic plants (Hiatt et al. 1989), the secretion of biologically active blood substitutes, namely, human interleukin-2 and interleukin-4 in transgenic tobacco suspension cultures (Magnuson et al. 1998), the expression of nopaline synthase-human growth hormone chimeric gene in transgenic calli of tobacco and sunflower (Barta et al. 1986), human interferon (De Zoeten et al. 1989), and human serum albumin (Sijmons et al. 1990). The successes of molecular farming (extensively reviewed by Kumar et al. 2007) that involved the transfer of the desirable gene to an appropriate host system, optimization of the desirable pattern of gene expression, and optimal recovery of the recombinant protein in the form of a pharmaceutical product peaked with the achievement of oral immunization with a recombinant bacterial antigen produced in transgenic plants (Haq et al. 1995).

10.3 The Basic Goals and Strategies of Metabolic Engineering

10.3.1 Biochemical Pathways

The progress of metabolic engineering is closely related with the discovery and understanding of biochemical pathways. The more detailed and well-documented knowledge on the pathway of interest is known, the better chance exists that the engineering of such a pathway will be predictable and successful. Unfortunately, despite decades of elucidating pathways in various organisms, our knowledge is still far from being complete. The main problem is to identify all enzymes that catalyze individual reactions within the pathway. The analysis is still difficult due to their instability, low amount, and/or low activity among others. On the other hand, the isolation and identification of secondary metabolites is easier by using labeling techniques although many experimentally unconfirmed intermediates still exist. With the progress of molecular biology, other approaches were involved to help with the determination of involved enzymes. Most of them are based on "knocking out" the gene by various techniques such as transposon tagging, TILLING, RNAi (for details see Chap. 1-6 of this volume) or amiRNA, and then identify which enzyme was affected. This is usually easy when the knockout gene is manifested phenotypically, but it may be a daunting task if otherwise.

10.3.2 Functional Genomics

Using functional genomics is just another way of elucidating biochemical pathways starting from genes down to the proteins. Functional genomics is the usage of statistical methods and bioinformatics to determine the function of the genes (as the name suggests). It is obvious that the genomic sequence must be known in order to use this method. For time being there are not many sequenced plant genomes

available but this will change in the near future; a number of nonmodel plant species are now being sequenced and more are planned in the near future. Functional genomics utilizes the "–omics" family tools, e.g., transcriptomics, proteomics, and metabolomics. The tool of metabolomics is particularly important because it qualitatively and quantitatively analyzes all metabolites in the organism. When this is combined with the transcriptomics and proteomics, the complete picture can be seen, e.g., involvement of regulatory and structural genes in the organism (for details see Chaps 2–10 of Volume 2 of this series).

10.3.3 Compartmentalization, Transport, and Storage

Engineering a metabolic pathway in plants needs to be calculated with intra- and/or intercellular compartments. The genes need to be expressed in the correct compartment and in the correct type of cell. If not, the expression system may not work, or only with low yield, or the product may even have a toxic effect. Thus production of metabolites occurs in intracellular compartments such as vacuoles, endoplasmic reticulum, cytosol, plastids, etc., whereas intercellular compartments cover various plant tissues. The existence of compartments requires the existence of various transport systems as the intermediate metabolite must be quickly moved between different intra- and intercellular compartments. A number of transport mechanisms were described in the literature. It is a rather complex process, and thus the biochemical reaction rate may be also limited on the level of transport. Intermediate metabolites are usually stored in vacuoles and thus transport mechanisms are required for an import.

10.3.4 Basic Strategies

Early experiments to increase the yield and productivity of secondary metabolite production relied on enzyme modification. These qualitative principles are based mainly on the view that control of pathways must reside in relatively few enzymes whose in vitro properties suggest that they could be controlling flux in vivo. However, manipulating enzymes considered to be "rate limiting" has rarely had the expected outcome. Metabolic pathways have evolved to exhibit control architectures that resist flux alterations at branch points. Stephanopoulos and Vallino (1991) therefore introduced the concept of flexible and rigid nodes. The rigidity of the biochemical network or its resistance to variations in metabolic change is due to control mechanisms established to ensure balanced growth. The more rigid the branch point, the harder it is to increase the flux through one of its branches. For an engineering strategy to be successful, a sound understanding of the host cell is necessary to determine the types of genetic modifications needed to achieve the final goal. Some of the physiological considerations that should be examined

include the effects of genetic manipulation on growth and possible effects on unrelated systems. Traditional metabolic biochemistry did not provide the understanding needed to do this because it dealt with metabolic regulation in terms of a few qualitative principles and was thus sometimes called "reductionist approach." Recently, a fast-growing field was introduced in the biological research referred as "systems biology." As the name suggests systems biology aims at systems-level understanding, as distinct from understanding individual system components such as particular genes or enzymes (Kitano 2002). At the very core of systems biology is the goal of being able to model a living organism. The systems range from metabolic pathways and gene-regulatory networks all the way up to whole cells and organisms. Thus it offers the –omics integration together with phenotypic data for studying plant organisms and even their interaction within their ecosystems. As for metabolic engineering, the ultimate aim is to use the comprehensive experimental data sets describing changes in transcripts, proteins, metabolites, and flux to generate a complete mathematical description of the metabolism of a model plant species. It is envisaged that such a model would allow a truly predictive engineering of plant metabolism. This is an ambitious aim that will require a sustained commitment of resources and unprecedented technological developments to be achieved (Sweetlove et al. 2003). Within system biology it is possible to establish theoretical basis for determining which enzymes should be manipulated to achieve a desired outcome. One such theoretical basis is metabolic control analysis (MCA) which plays a central role in metabolic engineering.

Unlike traditional biochemistry, MCA is based upon the fact that a single rate-limiting step may not exist and several steps may share control of the metabolic network. Three commonly used normalized sensitivity measures, referred as Control Coefficients that quantify how the control of steady-state fluxes and concentrations is distributed between different reactions in a metabolic network, have been defined. Control Coefficients refer to the whole metabolic pathway (i.e., they are systemic or global properties). A Control Coefficient is a relative measure of how much a perturbation to, for example enzyme activity, affects a system variable, e.g., a flux or metabolite concentration. Flux Control Coefficient is the heart of the theory. It is a measure of how a change in the concentration of the enzyme affects the steady-state flux through that particular enzyme. That is, it is the measure of the degree of control exerted by enzyme on this steady-state flux. The Concentration Control Coefficient is a measure of the extent of control exerted by the enzyme on the steady-state concentration, while the Elasticity Coefficient is a measure of the response of the reaction rate upon changes in the concentration. Thus it refers to properties of individual enzymes in the pathway (they are local not systemic properties, and are related to classical enzyme kinetics).

Three main types of metabolic engineering based on MCA can be considered (Bailey 1991):

1. Extending an existing pathway to obtain a new product
2. Amplifying a flux-controlling step
3. Diverting flux at branch points ("nodes") to a desired product by:

(a) Circumventing a (feedback) control mechanism
(b) Amplifying the step initiating the desired branch (or the converse)
(c) Removing reaction products
(d) Manipulating levels of signal metabolites

These strategies have one major disadvantage: detailed knowledge of the network pathways and enzyme kinetics must be available. In contrast, the concept of inverse metabolic engineering does not require such knowledge. It is based on first obtaining the desired phenotype and later to determine environmental or genetic conditions that confer this phenotype, and finally to alter the phenotype of the selected host by genetic manipulation (Bailey et al. 1996; Delgado and Liao 1997).

10.4 Engineering Primary Metabolic Pathways

10.4.1 Carbohydrate Metabolism

In plants, the process of photosynthesis results in the production of sugars as direct products that undergo reversible conversion into storage carbohydrates such as starch and fructans, and the structural carbohydrate, cellulose (Fig. 10.1).

Starch as a storage carbohydrate accumulates transiently in leaves and stably in seeds, tubers, and roots. The importance of starch as a stable dietary carbohydrate and its many industrial uses render it as a favorite target for metabolic engineering in attempts to increase starch yields by changing the relative proportions of its structural components, amylose and amylopectin. Modulating the enzymes controlling starch synthesis and branching in potatoes resulted in the production of high-amylose starches that have important domestic uses such as improved frying and industrial uses as gelling agents and thickeners, and high-amylopectin starches notable in their use for improved freeze thaw characteristics, improved paper quality or adhesive manufacture (Capell and Christou 2004). Antisense expression of the *Waxy* gene in transgenic rice resulted in low-amylose rice grains of improved

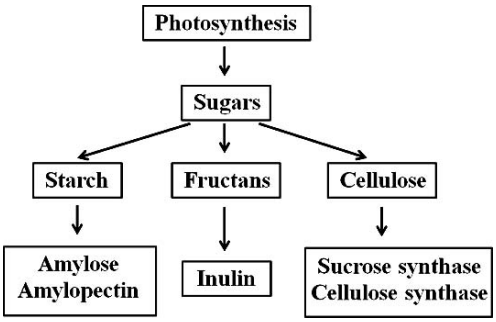

Fig. 10.1 Targets of carbohydrate metabolism

quality (Liu et al. 2003). Fujita et al. (2003) developed transgenic rice with modified amylopectin by antisense expression of the gene encoding isoamylase. Vincken et al. (2003) used a starch-binding domain from potato-granule bound starch synthase I to target luciferase to the inside of a starch granule, a promising method for directing recombinant enzymes into the starch granule for starch metabolic engineering. Novel starches were produced by changing the nature and frequency of branching directed toward a more versatile α-glucan synthesis by using bacterial enzymes (Kok-Jacon et al. 2003). Seed-specific overexpression of the potato sucrose transporter in transgenic pea was shown to increase sucrose uptake and growth rates of developing cotyledons (Rosche et al. 2002). Regierer et al. (2002) demonstrated that by increasing the activity of plasticidal adenylate kinase in transgenic potato, a larger pool of adenylates become available to fuel several metabolic pathways. This resulted in a 60% increase in overall starch levels, a two- to four-fold increase in amino acid levels combined with an increased tuber yield. Transgenic wheat and rice transformed with modified maize ADP-glucose pyrophosphorylase (*shrunken2*) targeting the enhancement of the enzyme activity in wheat endosperm and deregulation of the enzyme in rice endosperm resulted in a 40% and 20% increase in seed biomass, respectively, compared to wild-type controls (Smidansky et al. 2002, 2003).

The ability of fructans to substitute as low-calorie alternatives to fats due to their similar texture as fats attracted their attention in metabolic engineering. Inulin from chicory is a commercially available fructan. Bacterial and plant enzymes for fructan biosynthesis have been introduced into several crops to facilitate the large-scale extraction of fructans (reviewed in Ritsema and Smeekens 2003). The importance of fructans in protection of plants from abiotic stresses prompted their use in producing improved transgenic plant varieties by metabolic engineering (reviewed in Chen and Murata 2002).

The importance of the structural carbohydrate, cellulose, as pulp and fiber, and its role as a starting material for commercially important polymers make it an attractive target for metabolic engineering. Though the complete biosynthetic pathway of cellulose is not worked out, certain important enzymes involved in the process have been identified and exploited in metabolic engineering. Suppression of sucrose synthase gene expression was found to repress cotton fiber cell initiation, elongation, and seed development (Ruan et al. 2003). In *Arabidopsis*, the functional analysis of the cellulose synthase genes *CesA1*, *CesA2*, and *CesA3* revealed their role in primary and secondary cell wall formation (Burn et al. 2002), and the expression of a mutant form of cellulose synthase AtCes47 caused a dominant negative effect on cellulose biosynthesis (Zhong et al. 2003).

10.4.2 Amino Acid Metabolism

Metabolic engineering specifically targeted toward increasing the content of essential amino acids such as lysine, threonine, methionine, and tryptophan in

food crops remains an exciting proposition (Galili and Hofgen 2002). More recently, metabolic engineering complemented by RNA interference (RNAi) resulted in effective protocols for multiple point intervention in well worked out amino acid pathways. A good illustration of this strategy can be seen in generating a novel opaque variant of maize by a single dominant RNAi-inducing transgene targeting the zein genes (Segal et al. 2003). This novel version of *opaque2* has increased lysine content. Increasing lysine content was also demonstrated in transgenic *Arabidopsis*, where multipoint engineering of lysine metabolism was achieved by combining the overexpression of a bacterial enzyme, dihydrodipicolinate synthase (DHPS) that is resistant to lysine inhibition together with knockout of lysine catabolism pathway (Zhu and Galili 2003). Whether either strategy was used alone, a 12-fold or five-fold increase in lysine content was observed, and when transgene expression and knockout were in combination, an 80-fold increase was observed. Yet another valuable amino acid for metabolic engineering is proline as its role in plant stress responses makes it an important target for modulation to generate transgenic stress tolerant plants (Chen and Murata 2002).

10.4.3 Polyamine Metabolism

Polyamines are low molecular weight polycationic molecules that are known to play an important role in plant defense and in the regulation of plant growth and development (Rajam 1997; Kumar et al. 2006). These small aliphatic amines are derived from the amino acids, ornithine and arginine, by a decarboxylation pathway (Bhattacharya and Rajam 2007). Three major polyamines found in plants are putrescine, spermidine, and spermine. Putrescine is produced by the decarboxylation of arginine catalyzed by the enzyme arginine decarboxylase (ADC) or as in fungi by the decarboxylation of ornithine catalyzed by ornithine decarboxylase (ODC). Putrescine then acts as a precursor for the higher polyamines, spermidine and spermine, the conversion catalyzed by spermidine and spermine synthases, respectively. The reactions proceed by the addition of propyl amino groups to the decarboxylated S-adenosylmethionine (SAM) that is generated from SAM by SAM decarboxylase (SAMDC). A diamine cadaverine that is a penta homolog of putrescine is mainly found in legumes and is produced by the decarboxylation of lysine catalyzed by lysine decarboxylase (LDC) (Rajam 1997). The polyamine biosynthetic pathway is thus very well worked out and offers an array of possibilities for metabolite manipulation (Fig. 10.2). The corresponding genes for all the enzymes have been cloned in plants, namely the *adc* gene for tomato (Rastogi et al. 1993), pea (Perez-Amadour and Carbonell 1995), *Arabidopsis* (Watson and Malmberg 1996), and rice (Chattopadhyay et al. 1997); the *odc* gene from *Datura* (Micheal et al. 1996), tobacco (Mallik et al. 1996), and tomato (Alabadi and Carbonell 1998); the *samdc* gene from *Arabidopsis*, *Datura*, potato (Taylor et al. 1992), *Catharanthus* (Schroeder and Schroeder 1995), tomato, tobacco (Kumar et al.

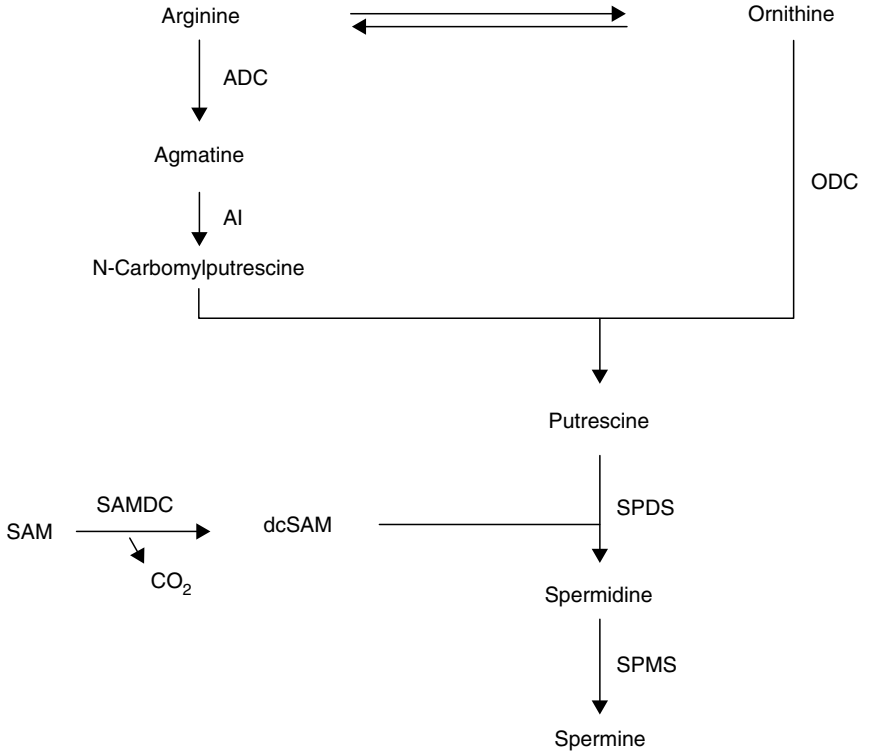

Fig. 10.2 Polyamine biosynthetic pathway in plants. ADC, Arginine decarboxylase; AI, Agmatine iminohydrolase; ODC, Ornithine decarboxylase; SAM, S-adenosyl methionine, SAMDC, SAM decarboxylase; dcSAM, decarboxylated SAM; SPDS, Spermidine synthase; SPMS, Spermine synthase. Reproduced from Bhattacharya and Rajam (2007)

1997; Park et al. 1998), and rice (Li and Chen 2000); and the *spd synthase* from tobacco, *Arabidopsis*, and *Hyoscyamus niger* (Hashimoto et al. 1998).

Metabolic engineering of polyamines has mainly utilized overexpression and antisense techniques. Overexpression of carrot *samdc* in rice showed increased levels of spermine and spermidine in seeds, and only spermine in leaves (Thu-Hang et al. 2002). The expression of yeast *samdc* in tomato under the control of a ripening-inducible E8 promoter increased spermidine and spermine levels in the fruit, and resulted in enhanced production of lycopene, a longer vine life, and nutritionally improved tomato juice (Mehta et al. 2002). The antisense suppression by an oat *adc* gene in rice reduced the putrescine and spermine levels, but no concomitant changes were observed in the downstream genes in the polyamine pathway (Trung-Nghia et al. 2003). Polyamines also act as precursors to many secondary metabolites and are thus important sources for engineering of secondary metabolic pathways.

10.4.4 Lipid Metabolism

The manipulation of oils and lipids in plants to change the quantity and nutritional quality of the plant fatty acids has far-reaching applications in food industry and human health as oils and fats are an important source of energy for the human body and form a vital component of many cell constituents. Since the main sources of fat in the human diet are vegetable oils, mostly soy, canola (oilseed rape), palm, peanut and sunflower, an attractive area of research has been the production of oilseed plants engineered to produce omega-3 long chain polyunsaturated fatty acids (LC-PUFAs) that have multiple health benefits in terms of cardiovascular and mental health. Attempts in this direction were deemed important in a quest to provide a successful alternative to that of the LC- PUFA-rich fish oils that proved to be undesirable food ingredients due to the increase in the vegetarian movement, the associated objectionable flavors, and more recently the rise in chemical and environmental contaminants in marine life that are difficult and cost prohibitive to remove from the fish oils. Improving the fatty acid content of plants has important industrial applications in production of detergents, fuels, lubricants, paints, and plastics. Our review touches on some of the important examples. For a more detailed exploration, the reader is directed to the many extensive reviews in this area published in recent years (Murphy 2002; Drexler et al. 2003; Singh et al. 2005; Damude and Kinney 2007).

Metabolic engineering of fatty acids is of great interest both at the laboratory and industrial research levels as even the most extensive modifications have no notable effect on the normal growth and development of the modified plant (Thelen and Ohlrogge 2002). Most higher plants have the ability to synthesize the main C18-PUFA, linoleic acid (LA), and α-linolenic acid (ALA), and to a lesser extent, γ-linolenic acid (GLA) and stearidonic acid (SDA). The inability of plants to elongate and desaturate these C18-PUFA acids into the beneficial LC-PUFA therefore makes it imperative to genetically engineer the genes that encode the required biosynthetic enzymes to convert the LA into an ω6 LC-PUFA like arachidonic acid (AA) or the ALA into an ω3 LC-PUFA like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (for details of the steps in this pathways, see Singh et al. 2005 and Fig. 10.3).

Initial attempts to engineer plant fatty acid profiles focused on the redirection of fatty acid biosynthesis in the developing seed by blocking fatty acid desaturation resulting in a high oleic soybean (Kinney et al. 1998), and the introduction of enzymes that redirected fatty acid synthesis to new end products, such as medium chain fatty acid oils resulting in high laurate canola (Del Vecchio 1996). These technically successful experiments involving the introduction of one or two transgenes were aimed at improving the oxidative stability of the oil without hydrogenation. Liu et al. (2001) transformed the oilseed rape, *Brassica napus* with cDNAs encoding desaturation enzymes, 18:1 Δ12 desaturase alone or in combination with 18:2 Δ6 desaturase resulting in seeds producing 46% ALA and 43% GLA, respectively. Han et al. (2001) engineered a 60% erucic acid producing oilseed rape

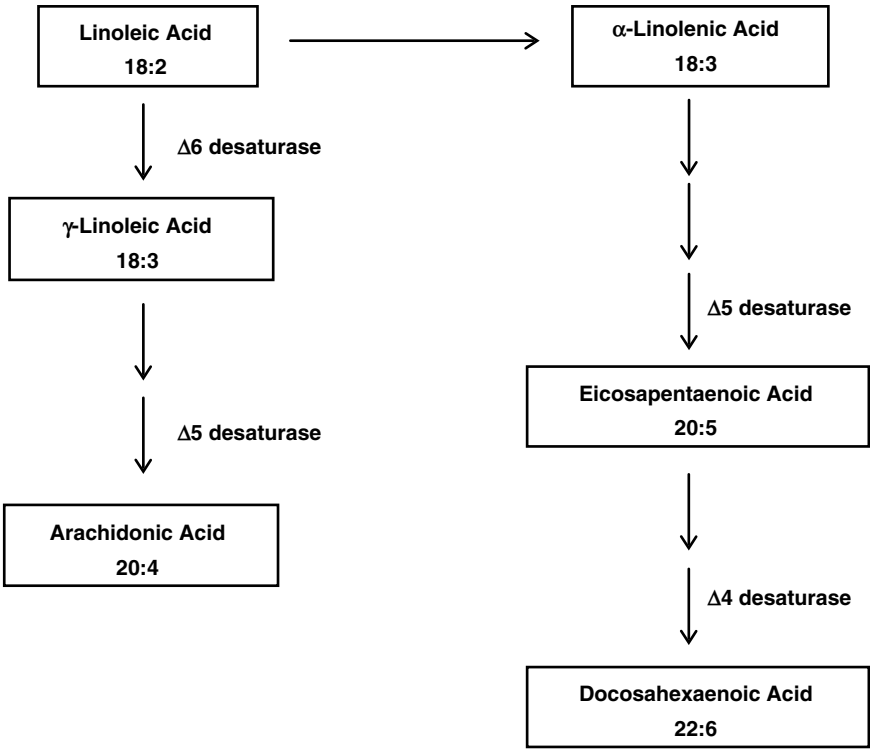

Fig. 10.3 Crucial steps in lipid metabolism of particular interest to plant metabolic engineers

by the combined expression of β-ketoacyl-CoA synthase and 22:1 acyl-CoA: lysophosphatidic acid acyltransferase. Anai et al. (2003) engineered rice with a soybean *FAD3* to increase the seed oil quality with a ten-fold increase of GLA. Sato et al. (2004) engineered marker-free soybean with levels of GLA as high as 50% by seed-specific expression of borage Δ6 desaturase gene.

With the advent of knowledge in gene discovery and corresponding genomic technologies, coupled with the spurt in elucidating gene expression pathways, the field of metabolic engineering forayed into technologies involving more complex manipulations of plant cell lipid metabolism involving engineering entire pathways in a single experiment. The first report of LC-PUFA production in higher plants as a "proof of concept" was by Qi et al. (2004) who demonstrated the increased synthesis of AA (6.6%) and EPA (3%) in *Arabidopsis* leaves by the transgenic expression of the individual genes in the Δ8 LC-PUFA pathway. The first successful reconstitution of Δ6 pathway for LC-PUFA production in plants and the first demonstration of LC-PUFA accumulation in seed oils were achieved in linseed (Abaddi et al. 2004). The most impressive demonstration of commercially significant concentrations of LC-PUFAs (19.5% of EPA) in plant seeds was achieved by placing the Δ6 pathway under the control of strong, seed-specific promoters in

soybean (Kinney et al. 2004) where, apart from the minimal set of genes, coding the Δ6 pathway was engineered in combination with the Arabidopsis *Fad3* (Yadav et al. 1993) and the *Saprolegnia diclina* Δ17 desaturase (Pereira et al. 2004). In a further improvement of this technique, Damude and Yadav (2005) utilized the Δ15 desaturase from *Fusarium moniliforme* (Damude et al. 2004) instead of the *Fad3* to generate soybean plants with an overall 57% increase of ω3 LC-PUFA content.

10.4.5 Metabolic Engineering in Chloroplast Genome

The genetic engineering of plastids started in 1990, when Pal Maliga's lab demonstrated the first successful and stable transformation of plastids in higher plants (Svab et al. 1990). At that time, few scientists envisaged the use of plastids in metabolic engineering. However, 5 years later Maliga's lab published another paper describing the expression of *Bacillus thuringiensis* cry toxin in chloroplasts. Although this toxin was very difficult to express in a plant's nucleolar genome, expression in chloroplasts was shown to be at extraordinary levels (McBride et al. 1995). This article generated great interest among biotechnologists. Together with other advantages, such as lack of epigenetic processes and gene silencing, the possibility to use precise homologous recombination for transgene integration and lack of pollen transmission, plastids promise to become great tools in metabolic engineering. Moreover, plastids integrate and express foreign sequences as operons (Ruhlman et al. 2007) and the ability of plastid expression system to transcribe operons from a single promoter, and thus enabling the expression of multiple genes in a single recombination event, makes possible the expression of multienzyme pathways in the first transformed generation eliminating the need to cross lines recombinant for individual genes (Quesada-Vargas et al. 2005). These important properties make them an attractive alternative to nuclear genomic manipulations.

As mentioned earlier, the first use of plastids was in resistance engineering. Chloroplasts proved to be very suitable for expression of *B. thuringiensis cry* genes. Because of their different (prokaryotic) codon usage, expression from nuclear genome proved to be severely hampered. There is no need to adjust codon usage when expressed in chloroplasts (McBride et al. 1995; Kota et al. 1999). The expression efficiency was so high that cry protein crystals could be seen within chloroplasts (Daniell et al. 2001). Recently, the first insect-resistant soybean plants were generated, thus demonstrating that this technology works not only in tobacco model plant but also in important crops (Dufourmantel et al. 2005). Another example of resistance engineering is generation of glyphosate-tolerant plants. Glyphosate is a broad-spectrum herbicide, which blocks plant aromatic amino acids synthesis by competitively inhibiting the key enzyme 5-enol-pyruvyl shikimate-3-phosphate synthase (EPSPS). Thus, overexpression of EPSPS in plastids could block the inhibition effect of glyphosate. This presumption proved to be valid, as Ye et al. (2001) showed that chloroplast expression of an EPSPS gene yielded plants resistant to high doses of glyphosate.

Unlike resistance engineering, metabolic engineering does not require massive overexpression of the intermediate; to the contrary, this may even be disadvantageous. In plastids, therefore, expression mechanisms must be optimized by adjusting plastid expression signals. Unfortunately, this is likely to be a tedious process because the sequence of the coding region of the foreign gene itself often influences the accumulation of the expressed foreign protein and adjustment therefore relies on trial and error. On the other hand, the ability to engineer multiple genes (Daniell et al. 2005b), high levels of recombinant protein accumulation (Daniell et al. 1997), and the security of transgene containment due to maternal inheritance of plastid genomes in most crop species (Daniell 2002) are among the features that make the chloroplast system an efficient platform for metabolic engineering.

The first demonstration of the feasibility for engineering nutritionally important biochemical pathways in nongreen plastids was plastid expression of a bacterial lycopene β-cyclase gene in tomato chloroplasts (Wurbs et al. 2007). This resulted in herbicide resistance and triggered conversion of lycopene, the main storage carotenoid of tomatoes, to β-carotene (pro-vitamin A), an essential antioxidant. This yielded a four-fold enhancement in provitamin A content of the fruit. Thus far, the most complex and novel metabolic pathway introduced into tobacco plastids was that for the synthesis of the bioplastic polyhydroxybutyrate (PHB) from *Ralstonia eutropha* (Arai et al. 2001). Various techniques were used, of which the most promising appears to be plastid transgene expression using a nuclear-encoded and plastid-targeted ethanol-inducible T7 RNA polymerase promoter as this circumvents the toxic effect of constitutively expressed bacterial *phb* operon (Nakashita et al. 2001; Lossl et al. 2003).

Undoubtedly, the greatest importance for plastid metabolic engineering is the photosynthetic pathway where the *Rubisco* gene is their primary target. The work carried out in this field greatly exceeds the scope of this chapter; more than 5,000 manuscripts exist on this subject. Several extensive review articles were recently published covering this topic (Whitney and Andrews 2003; Bock and Khan 2004; Portis and Parry 2007).

Molecular pharming is the third category of plastid engineering. Extraordinary expression levels achieved in chloroplasts are undoubtedly the main reason for the high interest in the production of pharmaceuticals in plastids. For example, the tetanus toxin fragment was produced in tobacco chloroplasts with expression levels exceeding 25% of the total soluble protein (TSP) (Tregoning et al. 2003). Similarly, chloroplast expression of human serum albumin reached 11% of TSP (Fernandez-San Millan et al. 2003) and that for xylanase was 6% TSP (Leelavathi et al. 2004). Tobacco chloroplasts are able to correctly fold complex proteins with disulfide bridges, such as human somatotropin (Staub et al. 2000) and even full-size antibodies (Daniell et al. 2001). However, a significant increase in overall cost may arise if solubilization from inclusion bodies and refolding of these therapeutic proteins is necessary.

A disadvantage of using tobacco chloroplasts for protein production is that they are generally deficient in the capacity to glycosylate proteins since N- or O-glycosylation has a strong impact on the activity of several therapeutic proteins.

However, a recent discovery in *Arabidopsis* may remedy this situation. Villarejo et al. (2005) showed that a chloroplast-located protein in higher plants takes an alternative route through the secretory pathway and becomes N-glycosylated before entering the chloroplast. The other disadvantage of using tobacco plastids (and tobacco itself) is the high content of nicotine and other alkaloids that must be removed from the final product, increasing the overall cost. The choice of organism may therefore shift to edible plants since human proinsulin was shown to be produced in transgenic lettuce plastids (Ruhlman et al. 2007), and Daniell et al. (2005a) suggested that carrot appears to be ideal for oral delivery of therapeutic proteins. Commercialization of the expression of pharmaceutical proteins in chloroplasts is evidenced by an agreement made between Chlorogen (who has patented technology) and Sigma-Aldrich Fine Chemicals to produce four different proteins in tobacco plants.

10.5 Engineering Secondary Metabolic Pathways

Plant secondary metabolite pathways are the major target for metabolic engineering. Plant produces an enormous amount of secondary metabolites which play important roles in plant physiology. Some of plant secondary metabolites confer resistance against pests and diseases while some are the constituents of flower color, food flavor, and polymeric lignin for structural support and assorted medical agents such as phytoalexins, phytoestrogens (e.g., isoflavones and coumestrols) or chemopreventive anticancer agents (e.g., resveratrol), or regulate the development of fat cells (e.g., catechins), antimitotic, antimalarial, antioxidant, and antiasthmatic activities.

10.5.1 Transcription Factors as Tools for Metabolic Engineering

Transcription factors are regulatory proteins that can act as activators or repressors of gene expression through sequence-specific DNA binding and protein-protein interactions, mediating changes in the levels of mRNA accumulation. The molecular entities that are involved in such interactions are chromatin remodeling proteins other than the general transcription machinery (Latchman 2003). In recent years, a flurry in the knowledge of elucidating the functions of an array of transcription factors showed that many impact the flux through metabolic pathways and, since they tend to control multiple pathway steps, they are fast emerging as powerful tools to control complex metabolic pathways in plants (Broun 2004).

The potential of transcription factors as tools for manipulating metabolic pathways was recognized in the pioneering work of Goff et al. (1990) involving the maize flavonoid pathway regulators COLORLESS 1 and RED that were shown to induce flavonoid gene expression and anthocyanin accumulation in transgenic

maize. Bovy et al. (2002) generated high-flavanol tomatoes by the heterologous expression of maize transcription factor genes *LC* and *C1* that resulted in an increased flux of flavonoid pathway throughout the fruit as opposed to expressing a chalcone isomerase gene that resulted in enhanced flavanol production only in the peel. This result proves that activating a pathway regulator as opposed to a pathway gene can induce metabolite accumulation in a tissue where most relevant enzymatic activities are insufficient. Transcription factors can also be valuable as discovery tools to identify enzymes and accessory proteins associated with complex pathways. This was effectively demonstrated by Broun et al. (2004) when they overexpressed an ETHYLENE RESPONSE FACTOR (ERF)-like transcription factor WAX INDUCER 1 (WIN1), that singly causes wax accumulation in *Arabidopsis*. When *WIN1* plants were examined by Northern and microarray analyses, an array of genes involved in wax biosynthesis, such as *CER1*, *KCS1* were seen to be up-regulated as were also other lipid biosynthetic genes and proteins involved in cellular trafficking. This shows that *WIN1* can be a useful tool to dissect molecular mechanisms underlying poorly understood, complex metabolic pathways. Transcription factors can also be utilized to downregulate pathway flux as shown by Kawaoka et al. (2000) who silenced a DNA-binding protein, *NTLIM1*, in transgenic tobacco that resulted in a dramatic reduction in lignin production due to a significant decrease in the expression of early phenylpropanoid pathway genes.

10.5.2 Flavonoids

Flavonoids are a large family of plant secondary metabolites synthesized from the phenylpropanoid pathway (Dixon and Steele 1999; Winkel-Shirley 2001). The biosynthetic pathway of flavonoids is the best characterized of plant secondary metabolites in terms of chemistry, biochemistry, genetics, and molecular biology (Harborne 1988, 1994; Stafford 1990; Winkel-Shirley 2001; Grotewold 2006). The knowledge of flavonoid biosynthesis and the important functions of flavonoid compounds in plants and in human nutrition have made flavonoids and isoflavonoids excellent targets for metabolic engineering.

In the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamate. The cinnamate 4-hydroxylase (C4H) catalyzes the hydroxylation of cinnamate to *p*-coumarate that is converted by 4-coumarate: coenzyme A (CoA) ligase (4CL) to *p*-coumaroyl-CoA. The flavonoid biosynthesis starts with the condensation of one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA to produce tetrahydroxychalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). Chalcone is isomerized to a flavanone by the enzyme chalcone isomerase (CHI) (Fig. 10.4).

Flavanones (e.g., naringenin) provide a central branch point in flavonoid biosynthesis. From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids as flavones, flavonols,

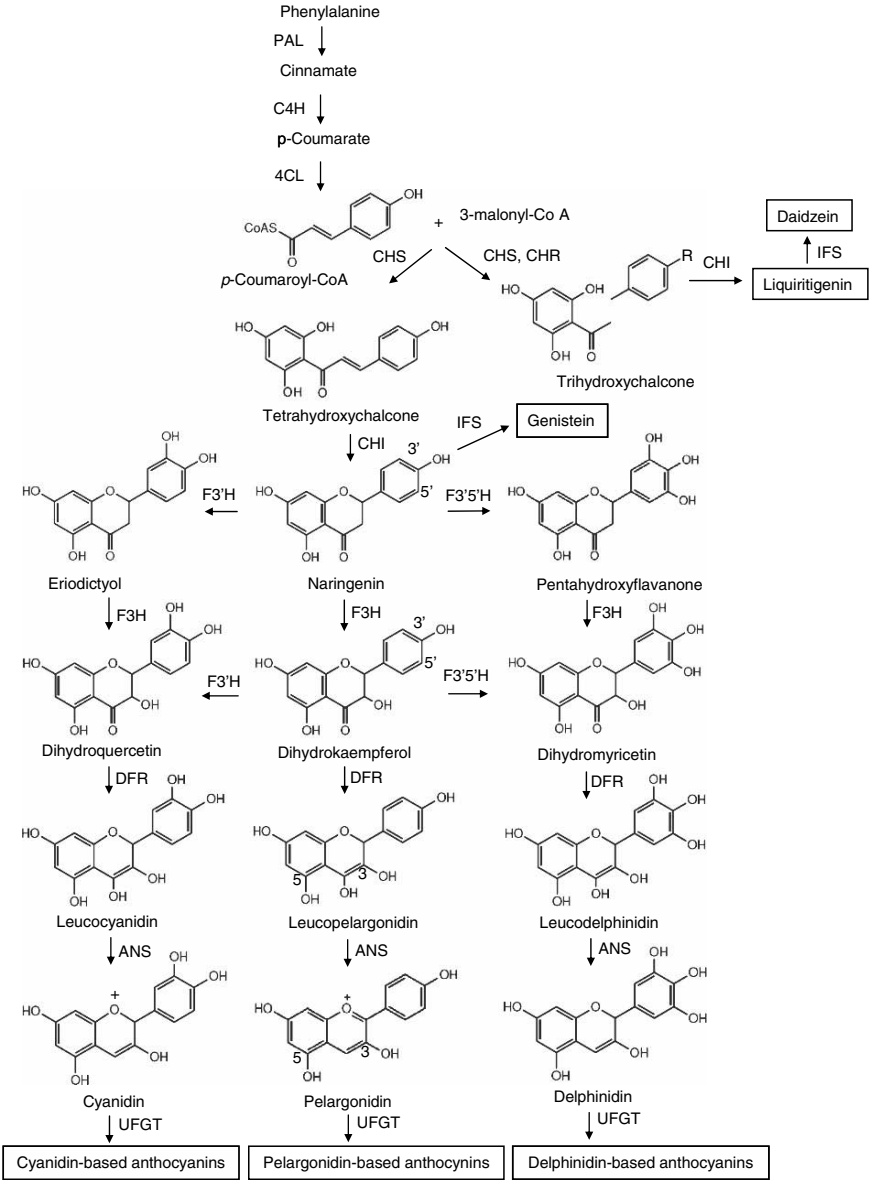

Fig. 10.4 A simplified biosynthesis pathway of isoflavonoids (daidzein and genistein) and cyanidin, pelargonidin and delphinidin-based anthocyanins. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:coenzyme A (CoA) ligase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′, 5′-hydroxylase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase and UFGT, UDP glucose:flavonoid 3-O- glucosyltransferase

isoflavones, anthocyanidins, and anthocyanins. Among these subclasses, isoflavones and anthocyanins are the main targets for metabolic engineering.

10.5.2.1 Isoflavone Biosynthesis and Metabolic Engineering

Isoflavones are mostly produced in the Papilionoideae subfamily of Leguminosae (Dewick 1994) such as soybean (*Glycine max*), green beans (*Phaseolus vulgaris*), and alfalfa (*Medicago sativa*). They are involved in plant defense mechanisms (Ebel et al. 1986; Rivera-Vargas et al. 1993; Graham and Graham 1996; Dixon and Sumner 2003) and symbiosis between the roots of leguminous plants and *Rhizobium* bacteria leading to the formation of nitrogen-fixing root nodules (Pueppke 1996; Spaink 2000; Ferguson and Mathesius 2003). Isoflavones have molecular structures similar to the human hormone estrogen and act as phytoestrogens. There are several reports of isoflavone activities important to human nutrition and medicine as anticancer and antioxidant compounds (for a review, see Ososki and Kennelly 2003; Cornwell et al. 2004).

The isoflavone phytoestrogens daidzein and genistein are synthesized from the phenylpropanoid pathway and stored in the vacuole as glucosyl- and malonylglucose conjugates (Graham and Graham 1996). The pathway to synthesize daidzein branches from the flavonoid biosynthesis catalyzed by chalcone synthase and a legume-specific enzyme, chalcone reductase (CHR) to generate trihydroxychalcone which is consequently converted to daidzein through reactions catalyzed by chalcone isomerase (CHI) and isoflavone synthase (IFS) (Fig. 10.4). Genistein synthesis from naringenin is mediated by IFS (Fig. 10.4). The soybean IFS is encoded by two genes, *IFS-1* and *IFS-2*, that have been cloned and examined in some detail by several groups (Akashi et al. 1999; Steele et al. 1999; Jung et al. 2000; Yu et al. 2000).

Metabolic engineering of isoflavones by increasing isoflavone levels in soybean and the introduction of isoflavone biosynthesis in nonlegume crops such as maize, wheat, or rice that do not naturally produce isoflavones has been a focus of research in recent years due to their significant roles in plant defense and in human medicine and nutrition. It has been shown that the level of genistein produced and accumulated in leaf and stem tissues of *Arabidopsis* transformed with soybean *IFS* (Jung et al. 2000) is enhanced when the phenylpropanoid pathway is activated by high UV-light (Yu et al. 2000). Genistein in IFS-transformed tobacco accumulates to higher levels in anthocyanin-producing flowers than in leaves. The production of genistein in maize black mexican sweet (BMS) cells required maize transcription factors C1 and R in addition to IFS. Further, BMS cells cotransformed with *IFS*, *CRC* (a chimeric transcription factor containing maize *C1* and *R* coding regions), and soybean *CHR* can produce the novel compound daidzein (Yu et al. 2000).

These results show that isoflavones can be synthesized in nonlegume plants, albeit at low levels compared to those in soybean. There are factors that limit the flow of intermediates toward isoflavone biosynthesis. These include flavanone

3b-hydroxylase (F3H), the major flavonoid enzyme that competes with IFS for the common substrate naringenin. Silencing of F3H reduced flavonoid biosynthesis and increased isoflavone accumulation. This was demonstrated in soybean lines transgenic for *CRC* as they accumulated isoflavones to much higher levels than in wild-type seed (Yu et al. 2003). Whereas the expression of *CRC* alone in soybean seeds gave only a small increase in isoflavone and flavonol levels, the coexpression of *CRC* together with a silencing construct targeting flavanone 3b-hydroxylase (F3H) resulted in increased total isoflavone content up four-fold higher than in wild-type seed. These high isoflavone soybeans would be useful for the production of soy foods providing potentially greater health benefits to consumers.

Another possibility for increasing the isoflavonoid content of nonlegume plants is by protein engineering. The expression of an IFS-CHI fusion protein in transgenic tobacco plants produced higher levels of the isoflavone genistein and genistein glycosides than plants transformed with IFS alone (Tian and Dixon 2006).

10.5.2.2 Anthocyanin Biosynthesis and Metabolic Engineering

Anthocyanins belong to the most important flavonoid class. They are major components of flower and fruit colors. The key enzymes to regulate the anthocyanin synthesis are flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′, 5′-hydroxylase (F3′5′H) that catalyze hydroxylation at the 3′- or 3′5′-positions of the B-ring of flavonoid compounds. F3′H has a wide substrate range and can convert the naringenin to eriodictyol, the dihydrokaempferol (DHK) to dihydroquercetin (DHQ), and the flavonol kaempferol to quercetin. F3′5′H catalyzes the hydroxylation of both 3′ and 5′ positions of the B-ring leading to the conversion of naringenin and DHK to pentahydroxyflavanone and dihydromyricetin (DHM), respectively (Fig. 10.4). These compounds are catalyzed by flavanone 3-hydroxylase (F3H) to colorless dihydroflavonols, either DHK, DHQ, or DHM that will be reduced by dihydroflavonol reductase (DFR) to leucoanthocyanins. These compounds are converted to the corresponding leucoanthocyanidins by anthocyanidin synthase (ANS). Anthocyanidins serve as substrates for anthocyanin. They are unstable and will couple to sugar molecules by enzymes such as UDP-glucose:flavonoid 3-O- glucosyltransferase (UFGT) to yield the final relatively stable anthocyanins (Bohm 1998; Harborne 1994).

Cyanidin-, pelargonidin-, and delphinidin-based anthocyanins are responsible for flower colors. Cyanidin-based anthocyanin is the source of red and magenta colors, whereas pelargonidin-based anthocyanin is responsible for orange, pink, and bright red colors, and delphinidin-based anthocyanin for violet and blue colors. Limited ranges of flower color for individual plant species reflect the absence, mutation, or abundance of genes involved in anthocyanin biosynthesis, substrate-specificity of key enzymes, and/or the temporal and spatial regulation of the anthocyanin biosynthesis.

Some plants such as roses (*Rosa hybrida*), chrysanthemums (*Chrysanthemum morifolium*), and carnations (*Dianthus caryophyllus*) do not produce purple delphinidin-based anthocyanins because they lack the activity of F3′5′H (Elomaa

and Holton 1994; Holton and Tanaka 1994; Tanaka et al. 1998; Mol et al. 1999). Petunia (*Petunia hybrida*), cymbidium (*Cymbidium hybrida*), tomato (*Solanum lycopersicum*), and cranberry (*Vaccinium macrocarpon*) do not produce brick red/orange pelargonidin-based anthocyanins because their dihydroflavonol 4-reductases (DFRs) have strict substrate specificities and cannot utilize DHK as a substrate (Forkmann et al. 1980; Meyer et al. 1987; Johnson et al. 1999; Polashock et al. 2002).

Transformation of the maize *A* gene coding for the dihydroquercetin-4 reductase (DQR) into the petunia mutant, which shows no flower pigmentation (Meyer et al. 1987), demonstrated that the DQR in transgenic petunia can reduce the petunia DHK to leucopelargonidin, which leads to the production of red color pigmentation. This indicated that it is possible to generate a novel flower color in plants by introducing the gene involved in anthocyanin biosynthesis pathway.

However in some plants, the introduction of a foreign gene may not be sufficient to convert the metabolic flux of anthocyanin biosynthesis to obtain a plant with the desired flower color. It is necessary to select the suitable plant cultivars that have the appropriate genetic background and flavonoid composition and/or the artificial down-regulation of a competing endogenous pathway (Tanaka 2006; Tanaka and Brugliera 2006). Florigene Ltd. (Melbourne, Australia) generated violet carnations by transforming the petunia *F3′5′H* gene in combination with the *dfr* genes into white carnation cultivars that specifically lacked the *dfr* gene (Mol et al. 1999; Fukui et al. 2003).

Metabolic engineering of rose flower color is more complicated than in carnation as in nature there is no white rose lacking the *dfr* gene. To solve this problem, Florigene Ltd. (Melbourne, Australia) and Suntory Ltd. (Osaka, Japan) employed the gene-silencing technique to switch off the *dfr* gene that produces the red pigment in rose. The *F3′5′H* gene from iris was then inserted into *dfr*-silenced rose to produce the blue pigment of delphinidin-based anthocyanins (Katsumoto et al. 2007). However, the blue rose generated by Florigene and Suntory is not a "true" blue, but it is in fact of a pale violet color. In addition to anthocyanins that determine the flower color, other factors such as vacuolar pH, copigments, metal ions, and anthocyanin modifications (acylation, glycosylation, and methylation) that influence the shade and intensity of flower color must be taken into account (Yoshida, et al. 1995; Yabuya et al. 1997; Mol et al. 1998; Tanaka et al. 1998). Anthocyanins are bluer in weakly acidic and neutral pH and they are redder in acidic pH. Rose petals are moderately acidic with a pH around 4.0, which inhibits the blue pigment, while the carnation petals are less acidic with a pH of 5.5. Several genes such as *ph1-ph7* and *Pr*, encoding the proteins that control the vacuolar pH, have been identified from petunia by transposon tagging and transposon display (Mol et al. 1998; Fukada-Tanaka et al. 2000; Quattrocchio et al. 2006; Verweij et al. 2008). It may be feasible to engineer true blue roses by manipulation of the anthocyanin genes and the transcription factors regulating their spatial and temporal expression.

The intra- or intermolecular stacking of copigments such as flavones, flavonols, phenylpropanoids, organic acids, and aromatic acylated groups also leads to a shift in the visible absorption maximum of the complex toward longer wavelength (bathochromic shift) (Goto and Kondo 1991). Inhibition of DFR in torenia

(*Torenia fournieri*) by antisense silencing of DFR increases the level of flavones, which made wild-type violet flower color more intensely blue (Aida et al. 2000). Cosuppression of flavone synthase II (FNSII) in torenia decreased the amount of flavones and increased the amount of flavanones, and yielded paler flower color and increased that of flavanones, generating transgenic flowers paler than wild-type ones (Ueyama et al. 2002).

Extensively studied copigments of flavonoids are in blue-flowered plants (Harborne and Williams 2000; Grotewold 2006) and the effect of metal ions associated with anthocyanins in flower (Kondo et al. 1992; Yoshida et al. 2003; Shiono et al. 2005; Shoji et al. 2007).

Anthocyanins form complexes with copigments such as flavones and flavanols by aggregation, resulting in shift of the visible absorption maximum of the complex toward longer wavelength (bathochromic shift). This usually leads to darker flower colors (Forkmann 1991). Flavones are common copigments that form complexes with anthocyanins. Mixtures of various molar ratios of anthocyanin fraction and flavone fraction from flower extracts of torenia (*T. fournieri*) were prepared in a pH 5.4 buffer and their visible absorbance was monitored. Mixtures with molar ratios corresponding to the endogenous concentrations in the petal had absorbance values corresponding to the color of the petals (Aida et al. 2000). In addition, antisense silencing of DFR in Torenia caused a marked increase in flavones, resulting in transgenic plants with bluer flower color than CHS-silenced plants (Aida et al. 2000). FNS genes that are responsible for the biosynthesis of flavones have been isolated from *Torenia hybrida* and other species.

Inhibition of DFR in torenia (*T. fournieri*) by antisense silencing of DFR increases the level of flavones, yielding a more intense blue flower color than that of the wild type (Aida et al. 2000). Cosuppression of FNSII in torenia reduced the amount of flavones and increased that of flavanones, generating transgenic flowers paler than wild-type ones (Ueyama et al. 2002).

Many metal ions including Cu^{2+}, Ca^{2+}, Al^{3+}, Fe^{3+}, Mg^{2+}, and Mo^{2+} were found to coexist with anthocyanins (Ellestad 2006). Such associations usually have a significant impact on flower color. For instance, addition of metal ions (Mo^{2+}) in vitro to purified anthocyanins from *Brassica rapa* can result in a color change from pink to blue (Hale et al. 2001). Energy-dispersive X-ray analysis showed that metal ions accumulated predominantly in the vacuoles of the epidermal cells. In *Brassica juncea*, the X-ray absorption spectrum of plant-accumulated Mo^{2+} was different than that for molybdate, and correlated with the cellular and subcellular distribution of water soluble, pH-dependent anthocyanins (Hale et al. 2001).

10.6 Future Roadmap

The increased interest in plant metabolic engineering in recent years can be attributed to four main factors: plants as a major source for medicinal products; plants containing health-promoting secondary metabolites; plants resistant to pests

and diseases; and plants with flowers of novel colors, patterns, scents. The rapid progress seen in successful and robust methods of plant genetic transformation (for details see Chaps 1-1, 1-2, 1-3, 1-4, 1-5, 1-7, 1-8 and 1-9 of this Volume) has revived the early promises of plant genetic engineering to provide many novel attributes to the world's flora.

10.6.1 Food for the World

The primary need of mankind for plants is to provide food security. As defined by Wikipedia, food security refers to the availability of food and its accessibility. A household is considered food secure when its occupants do not live in hunger or fear of starvation. It is hard for those of us living in well-developed nations that over 850 million of the world's 6.6 billion population people are chronically hungry and up to two billion people lack food security intermittently due to varying degrees of poverty (FAO 2003).

10.6.2 Biofortification of Plant-Based Foods

The requirement of a minimal daily intake of essential micronutrients, vitamins, and minerals for the maintenance of optimal human health has long driven the focus of plant science research toward combating micronutrient malnutrition by developing superior plant varieties with improved nutritional value. The approach evolved into what is now known as biofortification where efforts are on to deliver the daily micronutrients directly into the staple crops consumed by mankind. This approach was intended to alleviate the increased industrial costs incurred by the fortification of processed foods with the micronutrients. The dissection of plant metabolic pathways involved in synthesis of essential dietary micronutrients showed that all plants have the biochemical activities necessary to synthesize and accumulate a near full complement of essential dietary micronutrients with the exception of vitamins D and B_{12} (Dellapenna 2007). If we observe the dietary habits of populations with the maximum risk of micronutrient malnutrition, they consume foods like rice, wheat, cassava, and maize that contain insufficient daily intake levels of essential dietary micronutrients. These specific staple crops are being targeted by plant metabolic researchers for biofortification so that the levels of the limiting micronutrients in these crops can be increased by an effective combination of breeding and genetic engineering. The first successful and also the most popular example in this area included the biofortification of provitamin A in rice that resulted in the nutritionally enhanced "golden rice" that will help toward combating malnutrition-induced eye defects (Ye et al. 2000; Datta et al. 2003). The historical and scientific details of the steps involved in the golden rice technology are given in

Emani et al. (2008). Biofortification of the vitamin E family of lipophilic antioxidants called tocochromanols that protect against effects of free radicals, reactive oxidation species, and lipid oxidation was achieved in barley (Cahoon et al. 2003), soybean (Van Eenennaam et al. 2003), and oilseeds (Karunanandaa et al. 2005). Biofortification of another important B-vitamin, folate, was achieved in tomato fruit (Diaz de la Garza et al. 2007).

Initially, plant metabolic engineering involving manipulating nutritional levels relied on expression or silencing of a single gene in well-studied metabolic pathways that proved effective when the engineered step was at a potential metabolic branch point. Future research especially in the area of biofortification should consider expressing heterologous enzymes at such steps that can potentially create novel substrates for already existing enzymes that may lead to the creation of an entirely new branch in the pathway and formation of novel products (Kinney 2006).

10.6.3 Biofuel From Plants

Biofuels have come of age as attractive sources of energy around the world as finite petroleum reserves, increasing demands of energy in both industrially developed and rapidly industrializing countries combined with negative environmental effects of petroleum undermines both economic strength and threatens national security (Bordetsky et al. 2005). The biofuel that has the potential for extensive usage around the globe is ethanol due to its environmentally friendly nature owing to low toxicity and ready biodegradability, and the ability to be produced from the abundant biomass of land plants. Ethanol production from biomass also reduces the levels of greenhouse gasses. The usage of plants for cellulosic ethanol production as compared to other sources like starch and sugar-derived ethanol is because of the lower costs and abundance of biomass as compared to the limited supplies and the food supply competition related to starch and sugar. Food crops such as corn, rice, sugarcane, perennial grasses such as switchgrass and giant miscanthus and woody crops such as polar and shrub willow are potential sources for ethanol production (Sticklen 2008). Plant cell wall is the source for the lignocellulosic biomass, and the secondary cell wall contains cellulose, hemicellulose, and lignin (Sticklen 2008). Enzymatic hydrolysis utilizing cellulases and hemicellulases can convert the cell wall polysaccharides to fermentable sugars, the main barrier to overcome being the lignin that prevents accessibility of the enzymes to the polysaccharides. Lignin breakdown by chemical and heat treatments combined with microbial production of cellulases was the starting point in developing efficient processes to produce fermentable sugars for biofuels. Metabolic engineers can play a vital role in research aimed at characterizing the cell-wall deconstruction enzymes, especially in isolating enzymes that can resist higher conversion temperatures and a range of pHs during the pretreatments aimed at lignin hydrolysis that is one of the important challenges in cellulosic ethanol production (Sticklen 2008). Presently, the successes

seen in plant genetic transformation can be exploited to design strategies to express plant cell wall deconstructing enzymes in transgenic plants to enable cheaper processes for producing cellulosic hydrolysis enzymes (Sticklen et al. 2006). A comprehensive characterization of all the steps in cellulose biosynthesis (Kawagoe and Delmer 1997) has been the focus of plant molecular biologists (Arioli et al. 1998) now being complemented by the latest advances of genomics and microarray technology to identify the relevant useful genes (Persson et al. 2005; Andersson-Gunneras et al. 2006). This would enable the efforts to increase the plant cellulosic biomass in terms of increased cell-wall polysaccharide content by genetic manipulations. The increase seen in the overall plant biomass in rice by the elevated expression of ADP-glucose pyrophosphorylase by an endosperm-specific promoter (Smidansky et al. 2003) throws open the doors for metabolic engineers to explore manipulations of other enzymes of the starch biosynthetic pathway to aid in a shift to increasing biomass for biofuel production. A better understanding of the lignin biosynthesis aimed at down-regulation of the involved enzymes to modify structural components of lignin or reduce the lignin content itself is the need of the hour to avoid the need for the expensive pretreatments (Sticklen 2008). For a more exhaustive review of transgenic technology related to biofuels, refer to Chaps 2–6 of the Volume 2 of this series.

10.7 Conclusion: Factories of the Future

Plant metabolic engineering has had a fairly successful run in the academic and industrial circles, but a fact that cannot be ignored is that it was punctuated by several failures and limitations. Several "proof of concept" experiments successful in model plants failed to live up to expectations in the cultivars. The key to successfully overcoming such challenges is to fully exploit the advent of applied genomics, proteomics, and metabolomics to comprehensively understand poorly characterized metabolic pathways. The newly emerging discipline of systems biology should be used to see beyond the boundaries of metabolic pathways that are subject to engineering to create and understand the complete metabolite profiles in the plant world. The acquired knowledge will enable researchers worldwide to successfully dissect and understand metabolic pathways, and successfully increase their ability to both model and implement multipoint metabolic manipulations. This would in turn result in an avalanche of desirable products in transgenic plants that would be rightly called the "factories of the future." The advent of "molecular pharming" has readily identifiable benefits for mankind in a cost-effective, nutritionally wholesome, and environmentally sustainable manner. Together with enhancing the esthetic nature of the world through the development of novel ornamental plants, it can supplement the development of the still irreplaceable traditional agriculture to meet the rising food security in the centuries to come.

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