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Biotechnological Approaches to Barley Improvement

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Biotechnological Approaches to Barley Improvement

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Preface

Barley is deemed one of the first plant species to be domesticated. Its cultivation over a very broad range of environments and its versatile utilization for the production of raw material for feed and food render it a top-four crop plant worldwide. Representing the temperate cereals of the Triticeae tribe (wheat, rye, triticale), barley has been used as experimental model since the era of classical genetics and cytogenetics—a role that has been reinforced along the development of contemporary biology and crop plant research.

The generation of comprehensive genome sequence data is regarded as a cornerstone with major impact into all areas of modern barley research. Thus, we are convinced of the timeliness and importance to survey and reflect the current state of biotechnologically oriented barley research in the present volume of the Springer series *Biotechnology in Agriculture and Forestry*. Besides genome sequencing, major progress was achieved by deep sequencing-based transcriptomics, the establishment of comprehensive mutant panels and TILLING platforms, the generation of high-resolution genetic maps, efficient site-directed mutagenesis using customizable endonucleases, as well as the establishment of automated plant phenotyping facilities. This powerful suite of tools greatly facilitates the elucidation of molecular mechanisms underlying plant performance, which by itself is a prerequisite for knowledge-driven progress in barley breeding to cope with future challenges in agriculture and the related value chain.

The present volume is structured into two major sections: the first focusing on current agricultural challenges and approaches to barley improvement and the second giving insight to recent progress in methodology. The individual chapters provide the reader with comprehensive information spanning from fundamental aspects to special applications.

We are grateful to all the contributing authors—all are leading scientists in their respective field of research—for their outstanding input. All chapters were extensively peer reviewed to ensure for high standard and scientifically sound information throughout this compilation. We thus are very grateful to the contribution of all the involved reviewers, who were willing, in addition to the general load of

reviewing that all of us are facing these days, to review and comment on the contributions to this volume. All their names are listed overleaf and their help is very much appreciated. We hope this volume will serve as a useful resource for interested readers with good basic biological knowledge, students, and teachers especially of the life sciences as well as for established scientists working on barley or related subjects.

Great thanks also to Springer Publishing for its indefinite patience and support.

Gatersleben, Germany

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Part I
Agricultural Challenges and Approaches to Barley Improvement

Chapter 1
The World Importance of Barley and Challenges to Further Improvements

Harold Verstegen, Otto Köneke, Viktor Korzun, and Reinhard von Broock

1.1 Introduction

Some hold that mankind's most important invention is not the control of fire but rather the technology of fermentation. This opinion relates to a large extent to barley malt and beer production, a technology which enjoys a long tradition in many parts of the world. The earliest chemical confirmation of barley beer dates back to ca. 3400–3000 BC. As people settled more and more, domesticated cereal sources were used for food, of which barley is believed to be the first most common and therefore widely spread around the globe. During storage natural fermentation occurred which initiated the discovery of beer making (Michel et al. 1993). Already back then various flavors of light and dark beer were reported, drank by the poor and rich. Later, with continuous urbanization beer became a main source for beverage as populations lacked access to secure drinkable water. It was a safe drink for daily consumption while being less expensive than wine.

Although barley is still the main source for malt, the majority of the world's barley production [ca. 70 % based on FAOSTAT (2011)] is used for animal feeding. A small part finds its way directly into the human diet.

Good soil fertility and suitable climatic conditions are less important for barley than for the other major crops grown in the temperate zone like wheat and maize. And barley cropping is already economically viable at low levels of fertilization. Most other cereals need much higher input levels and production intensity.

Grain yield remains the most important breeding target, and over a long period of time, the contribution of breeding was such that it increased the yield by about 0.5 % per year (Comadran et al. 2010). In addition to grain yield, the main targets include yield stability, disease resistance, straw strength, winter hardiness (at least

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with respect to winter types), drought tolerance, and quality traits like protein and starch content as well as the specific quality traits needed for malting barley.

1.2 Worldwide Production and Usage

Barley was one of the first plants humans domesticated when switching from gathering and hunting to cultivation of land and animal husbandry 12,000–15,000 years ago (Badr et al. 1999). It is supposed that cultivated barley *Hordeum vulgare* L. was derived from *H. spontaneum* C. Koch, the only wild barley of more than 200 *Hordeum* species that can be crossed with *H. vulgare* L. (Nilan and Ullrich 1993).

Barley is a good choice as feed ingredient to provide the energy and dietary fiber intake of domestic animals. The largest consumer of feed barley is the pig industry. However, significant quantities are also consumed by dairy cows, goats, sheep, and camels. Industrial pig feed mixtures can contain as much as 60 % barley for early weaned pigs (Harrold et al. 1971) and as high as 80 % for fattening mature pigs (Kirchgeßner et al. 2011).

The estimated use of barley in 2011/2012 according to International Grains Council (2012) and FAOSTAT (2011) is 92 million tons for feed, 30 million tons for industrial use (mainly malt), about 8 million tons for sowing, and 7 million tons aimed as food for human use. Because of economic, climatic, and cultural influences, there are strong regional differences. For example, in Scotland more than 40 % of the barley is distilled (Reid 2009), and in Ethiopia hardly any barley is used for feeding at all (own observation).

Barley is grown on around 48 million hectares worldwide, thereby representing the fourth most widely grown cereal crop after wheat, maize, and rice (Table 1.1). Its major production areas are in those parts of Europe, Asia, North America, and Australia which share continental climatic conditions.

The mean current global grain yield is in the range of 2.7 tons/ha, although under more favorable conditions on-farm yields can reach up to 7 tons/ha (FAOSTAT 2011). The highest on-farm yield potential of winter barley observed by the authors was ~13 tons/ha, while spring type yields have topped 10 tons/ha. The world market for barley is expected to remain tight in the midterm future, with any rise in production almost certain to be offset by higher amounts used. Having decreased gradually over the past decade, consumption is projected to recover, mainly because of the anticipated rise in demand for feed in the context of restricted maize supply. The usage of barley as a feedstock for bioethanol, a practice more common in the EU than elsewhere, is expected to stay relatively minor. Meanwhile, the acreage of arable land devoted to barley production is expected to stay below the current 10-year average in Europe, Canada, and Australia, although it looks set to increase in South America. The current barley production is shown in Table 1.2. According to the International Grains Council (2012), the world's production in feed and malting barley is projected to increase by some 2 % per year.

Table 1.1 Barley production compared to other cereals in the world (FAOSTAT 2011)

Crop	Area (million ha)	Yield level (tons/ha)	Production (million tons)
Wheat	220.9	3.2	701.4
Maize	171.8	5.2	885.3
Rice	163.1	4.4	722.5
Barley	*48.4*	*2.7*	*133.0*
Sorghum	42.3	1.4	58.6
Oats	9.7	2.3	22.7
Rye	5.1	2.6	13.2
Triticale	3.8	3.5	13.5

Table 1.2 Barley production per continent and top 20 countries (FAOSTAT harvest 2011)

Region 2011	Area (million ha)	Yield level (tons/ha)	Production (million tons)
World	*48.4*	*2.7*	*133.0*
Europe	*24.4*	*3.3*	*81.2*
Russia	7.7	2.2	16.9
Ukraine	3.6	2.5	9.1
Spain	2.7	3.1	8.3
Germany	1.6	5.5	8.7
France	1.5	5.7	8.8
Poland	1.0	3.2	3.3
United Kingdom	1.0	5.7	5.5
Asia	*10.4*	*1.9*	*20.3*
Turkey	2.9	2.6	7.6
Iran	1.5	2.0	3.0
Kazakhstan	1.5	1.7	2.6
Syrian Rep.	1.3	0.5	0.7
Iraq	0.7	1.1	0.8
India	0.7	2.4	1.7
China	0.5	3.2	1.6
Northern America	*3.3*	*3.4*	*11.2*
Canada	2.4	3.3	7.8
USA	0.9	3.7	3.4
Australia	*3.6*	*2.2*	*8.0*
Africa	*4.9*	*1.3*	*6.5*
Morocco	2.0	1.1	2.3
Ethiopia	1.1	1.2	1.4
Algeria	1.0	1.5	1.5
South America	*1.2*	*3.2*	*3.8*
Argentina	0.8	3.9	3.0

1.2.1 Winter and Spring Type Barleys

The majority of the world's barley production is obtained from spring barley varieties. Spring barley varieties are generally known to have a broad adaption to different environments and do not require vernalization (a period of low temperature to stimulate flowering). Winter barleys, which are sown in the autumn, do need vernalization and can in general withstand environments with temperatures of as low as −20 °C (KWS LOCHOW GMBH own breeders observation). If conditions permit, winter barleys are preferred because of their yield advantage. As shown in Fig. 1.1, this extra yield performance reaches almost 2 tons/ha over the spring types under German and United Kingdom conditions. But in areas which experience low temperatures during a longer winter season, for example, Russia, the cultivation of spring varieties is essential.

Spring barley production dominates in Russia, Canada, Australia, South America, and Scandinavia. A successful spring cultivar needs to complete its growth cycle in a relative short time period of less than 5 months. Spring barley cultivars reach maturity in 90–120 days, which helps them to avoid summer drought. Robust cultivars can withstand a prolonged period of high temperature (up to 35 °C) late in their development without suffering major losses in grain yield (experience from KWS LOCHOW GMBH yield trials).

Spring barley is a nice alternative in a crop rotation system compared to spring wheat and maize that are both more demanding with regard to moisture and mineral

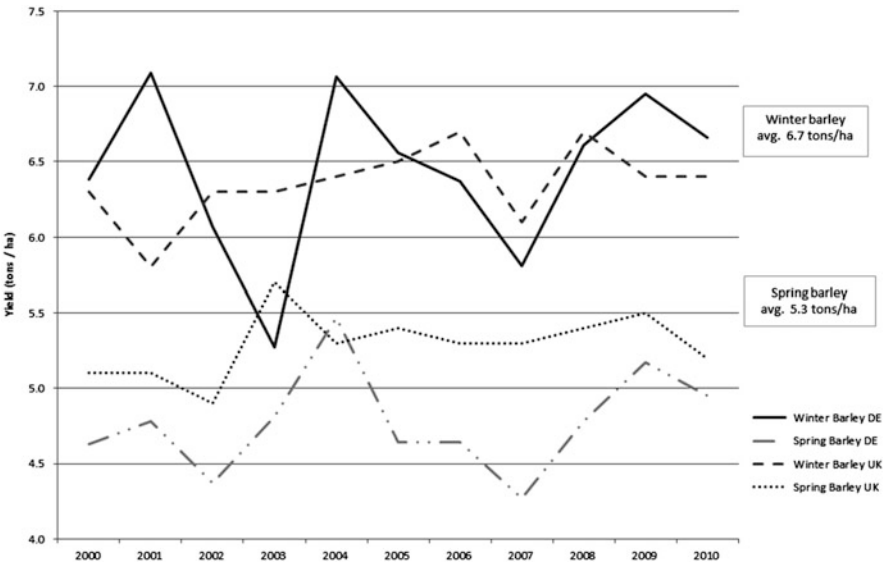

Fig. 1.1 The yield advantage of winter over spring barley during the previous decade in both Germany and the United Kingdom. *Sources:* statistic yearbook, Germany 2010; Farming-statistics@defra.gsi.gov.uk, 2011

fertility than barley and require constant moderate climatic conditions. Where low input agricultural farming and poor soil types normally limit the options, spring barleys do fit nicely. With the continuing rise in the world's population, there will be an increase in the intensity of farming, especially in more favorable arable areas (McCouch et al. 2013). As these optimal areas are getting scarce, though, there is a strong likelihood that spring barley will remain an important food and feed crop for the foreseeable future. With the current varieties, producers will need to ensure good farm practices that enable an adequate performance, with respect to both productivity and end-use quality despite of suboptimal growing conditions. At the same time, barley breeders are challenged to develop new cultivars allowing an economically viable production under these increasingly unfavorable conditions. This is a very important research objective, particularly as wheat and maize will almost certainly be the favored crop for those regions that have less stress and more preferred soils and climatic conditions.

Winter barley production may benefit from this resource competition for two reasons. First, climatic changes may open up new potential growing areas and extend the growing period of the crop in temperate regions by allowing planting before winter. This is interesting, especially as the resulting acceleration of development in early spring would support the avoidance of summer drought during the critical grain filling period (Olsen and Bindi 2002). Second, an extended growth area may result from future achievements in improving winter hardiness. This complex trait is a major challenge, largely because numerous stresses are involved, including wind chill, snow cover, diseases (in particular snow mold), nutrient supply, sufficient development before the winter, and late frost in spring (von Zitzewitz et al. 2011).

1.2.2 Two-Rowed and Six-Rowed Barley

The morphology of the barley ear, unique among the cereals, includes six-rowed as well as the ubiquitous two-rowed types (see Fig. 1.2). Two-rowed barley is a reduced six-rowed, in which the side florets are reduced and not fertile but still visible (for details see Komatsuda et al. (2007) and Chaps. 3 and 4). Two-rowed barley generally seems to have better kernel performance with high thousand kernel weight, slightly lower protein content, and, according to Bowman et al. (2011), higher starch content.

The proportion of the two-rowed types among cultivars has mostly historical reasons. Currently in Germany, the leading winter barley feed cultivars are all six rowed, while in the United Kingdom they are mostly two rowed. In France, most winter malting barley cultivars are six-rowed, while in the United Kingdom, Germany, the Czech Republic, and Poland, they are two rowed (KWS LOCHOW own data). Spring barley in Central Europe is almost exclusively of the two-rowed type, but in Norway, Finland, Canada, and the USA, a number of successful six-rowed spring barleys are cultivated.

Fig. 1.2 Two-rowed barley (*left*), six-rowed barley (*right*)

1.3 Breeding

1.3.1 Usage Types

1.3.1.1 Feed Barley

Most of the barley crop is used as livestock feed for predominantly cattle and pigs. The grain represents a favorable source of starch and has a higher content of crude fiber and protein than, e.g., maize (see Table 1.3). These favorable feed properties however pose a challenge to breeding. While the economic value focuses on high energy yield per hectare as well as yield stability under dynamic environmental conditions (with biotic and abiotic stress factors), the preferred levels of starch, protein, and fiber should be kept nevertheless. In addition, the agronomic traits like harvestability (lodging resistance, ear losses) and seed characteristics (full size kernel) are essential targets. Most of these traits are in a complex balance making it an optimization art to gain yield while maintaining or improving the other traits. As an example, the strong negative correlation between protein content and yield can be mentioned.

Important for its suitability as an animal feed type are also characters for digestibility. Optimal feed barley should have low acid detergent fiber (ADF) content for pigs, while at the same time the starch should have a low rumen digestibility for cattle. Most, if not all of these characters, are difficult to determine. They are therefore hardly used in large-scale selections up to now and mainly measured after a variety has been released on the market. This of course will

Table 1.3 Average nutrient composition of grains, dry matter basis (Ziegler 2012)

Grain[a]	Crude protein (%)	Starch (%)	DE[b] (Mcal/kg)	ADF[c] (%)	Ruminal starch digestion (% total starch)
Corn	10.3	75.7	4.1	3	65
Barley	*12.7*	*64.3*	*3.7*	*7*	*87*
Wheat	15.9	70.3	3.9	8	89
Rye	11.8	65.0	3.7	8	90
Triticale	15.7	67.0	3.7	8	90
Oats	11.6	58.1	3.4	16	92

[a]All grains were steam rolled, except corn which was cracked
[b]Digestible energy
[c]Acid detergent fiber

influence the success of a variety once experiencing the practical results on farm level.

1.3.1.2 Malting Barley

The second largest market is malting barley. Malting barley typically has a lower protein content (<11 %) than feed barley (>12 %). Compared to the feed types, the quality aspect is of utmost importance. Malting quality is a highly complex trait, reflecting the interaction between a number of overlapping but distinct sub-traits that are very important for the malting and brewing process. These processes adhere to strict quality and production standards demanding a balanced set of traits like malt extract, protein content, friability, soluble nitrogen content, viscosity, free amino nitrogen content, turbidity and color, α- and β-amylase activity, β-glucan and predicted spirit yield, and glycosidic nitrile content (Kunze 2010). These traits are used for variety testing by institutes like the Bundessortenamt in Germany, the Maltsters' Association of Great Britain, Newark, United Kingdom, and C.B.M.O. Comité Bière Malt Orge in France. An overview on these important malting quality parameters and their target values is shown in Table 1.4.

The traditional method for brewing beer, still largely followed in Europe and America, relies purely on malting barley as starch source, combined with the malting quality traits. Many quality beers follow the German "Reinheitsgebot," which demands to not use anything else, but barley malt, hops, yeast, and water to brew beer (Eden 1993). However, more and more other crops are used as sources for starch. In the growing markets like China, India, and Africa, more local sources like sorghum, rice, or maize are used. In general, these are cheaper starch sources, and with the improved developments on artificial enzyme mixtures, no "malting" enzymes of the crop are essentially needed anymore. However, as taste and color of the beer is still essential, the use of barley as starch source is still considered favorable.

In line with the innovations of the beer industry, the malting barley breeders have been able to adapt the varieties to the new demands. One example is the

Table 1.4 Malting quality—for good malt the following criteria must be reached (modified from Kunze 2010)

Quality parameter	Target values
Protein content	<10.8 %
Kolbach—or soluble protein	from 38 % to 42 %
Extract content	>82 %
Extract difference	from 1.2 % to 1.8 %
Viscosity	<1.55 mPa s
β-Glucan	<300 mg/l
Wort color	<3.4 EBC
Boiled wort color	<5.0 EBC
Nitrogen in malt (dry matter)	>0.65 g/100 g MTrS
Friability	>87 %
Viscosity 65 °C	<1.65 mPa s
β-Glucan 76 °C	<400 mg/l
DMS-P	<6 ppm

change of the traditional brewers' requirement for lower protein barley cultivars into a preference of a high amylase activity and high free amino nitrogen content of the malt (Kunze 2010). As shown in Fig. 1.3, the breeding efforts show a considerable improvement for malt extract.

Besides the changing quality demands which influence breeding targets, there is the trend to use winter barleys for the malting industry. The higher yield potential of winter over spring barley clearly indicated there was an opportunity. Consequently, in winter barley breeding in the last two decades, the emphasis has been directed on quality improvement. Breeding efforts have already improved the quality of winter

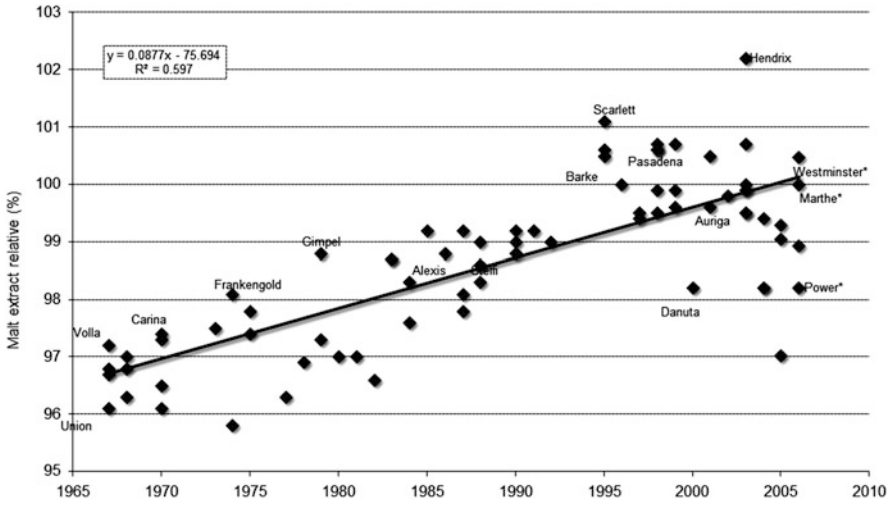

Fig. 1.3 Quality improvement of spring barley over the period from 1965 to 2005, as measured by malt extract (according to Herz 2012)

barley markedly. Depending on the market adoption from the maltsters and the farmers, winter cultivars could pick up some market share if they are of equivalent quality as current spring ones.

All in all, the malting barley market is a rather fragmented market sourcing different opportunities. Overall the worldwide consumption of beer is rising, while at the same time, the amount of barley malt per unit volume of beer brewed decreased due to the various innovations in the brewing industry. This development will likely continue and will also affect the malting barley market with even more fragmentation, both with respect to quality as well as regards the use of spring vs. winter types.

1.3.1.3 Food Barley

Barley grains are used as cooked whole or chopped grains after being de-hulled, similar to rice. In some areas of the world, barley is also milled, and the flour is mixed with other cereals and used for bread making. Barley contains β-glucan, which has been shown to reduce cholesterol (EFSA Journal 2011). It also has a low glycemic index and high fiber content which makes it a healthy choice for those with diabetes. Health, however, is not the main target in food barleys. More focus is put on taste, hullessness, and processing quality.

The breeding programs focusing on food source aspects are relatively small and mostly run by national agricultural research institutes and sponsored in an international context for a specific target. An example is described by Setotaw et al. (2012) where new food varieties are being developed for low-moisture areas in Ethiopia.

In most industrialized countries—as far as the authors know—there are no large breeding programs for barley specifically as food. There are a few small-scale specialized programs in some northern countries where taste, hullessness, and processing quality are characters of interest.

1.3.2 Disease Resistance and Plant Protection

Like all crops, barley is attacked by a number of pathogens, the three main pathogen types being fungi, viruses, and bacteria. Each pathogen targets a different physiological developmental stage of the barley plant, be it grain, stem, leaf, or ear.

In barley, the major diseases are powdery mildew (*Blumeria graminis*, formerly *Erysiphe graminis*, a fungus transmitted by wind from plant to plant), speckled leaf blotch (*Septoria passerinii*, a fungus often seen in hot spots), scald/leaf blotch (*Rhynchosporium secalis*, a fungus which spreads mainly by water splash dispersal), barley yellow and mild mosaic viruses (BaYMV and BaMMV, soilborne viruses transmitted by the plasmodiophorid *Polymyxa graminis*), net blotch (*Pyrenophora teres*, a fungus affecting mainly the leaf, usually coming from old

straw or stubble), and barley rusts like stem rust and leaf rust (*Puccinia graminis* resp. *Puccinia hordei*, both fungi).

Regionally, these diseases may differ in importance, depending on soil type, the prevailing agronomic production systems, and local climatic conditions. Especially for the fungal diseases, the infection pressure is highly depending on humidity combined with—depending on the fungus—cooler or warmer temperatures, which differs from year to year.

Additionally, some typically, rather regionally, observed diseases are spot blotch (similar to net blotch but reaction with spots instead of a net structure) and fusarium head blight (scab), barley yellow dwarf virus (BYDV, especially in North America, but also with increasing importance in France and Germany), Ramularia (leaf spot, northern United Kingdom), and yellow rust (France).

Major approaches to control diseases are (1) using resistant varieties, (2) using seed treatments, and (3) applying agronomic cultivation practices. Commonly a combination of all three is practiced. The development of resistant varieties is usually a long process, from finding resistant accessions to the introgression in the new variety. With the help of genetic resources, targeted evaluation, and modern genomic marker technology (see Sect. 1.3.4), new varieties are being developed. Examples are described by Jefferies et al. (2003), Scholz (2009), and Lüpken et al. (2013) showing the mapping and introgression of resistances for the yellow dwarf virus in barley. The process of screening, characterizing, and introgressing of new allelic variation from broad genetic resources will stay very important and probably gain importance as new viruses and fungal races that emerge continuously.

The use of grain treatment is widely practiced and very effective against diseases carried on or in the caryopses. In many cases, both soilborne and seedborne diseases can be completely prevented by effective chemical seed treatments. These treatments are usually also efficient to reduce the impact of leaf diseases from spores of neighboring fields as germination and early vigor of the host plant is stimulated.

The third factor is the professional use of agricultural practices like sanitation, crop rotation, generally recommended soil preparations (e.g., plowing under stubble), or sowing timing. Prior to sowing, before germination or in later stages, chemical treatments, especially fungicides, can be applied. The plant protection industry provides a range of products that differ in application, timing and repetition, target range, and compatibility with individual barley varieties.

1.3.3 Standard Breeding Methods

Barley is naturally a self-pollinating crop. At present, the overwhelming majority of barley varieties are based on pure line development. Hybrid breeding is also available and has resulted in the release of a number of hybrid varieties (Longin et al. 2012).

1.3.3.1 Development of Pure Line Varieties

For the development of pure line varieties, two basic breeding approaches are available: bulk selection and pedigree selection. Some modifications of the basic schemes have also gained importance, mainly the DH scheme. In all cases the starting point is to cross promising parental lines, usually called elite material, in order to find in their progeny such genotypes that combine the parents' quality and performance characteristics and ideally surpass the parents' performance in at least one of the traits of interest.

1.3.3.2 Pedigree Selection

In the pedigree scheme, selection starts directly after the first multiplication of the initial cross (in generation F2; see Fig. 1.4). Vigorous and healthy-looking individual plants are selected, and the progeny of a single F2 plant is grown as a single row ("F2 family") in the next generation. In the following generation (F3), selection first takes place between rows (families) and then between single plants within the row.

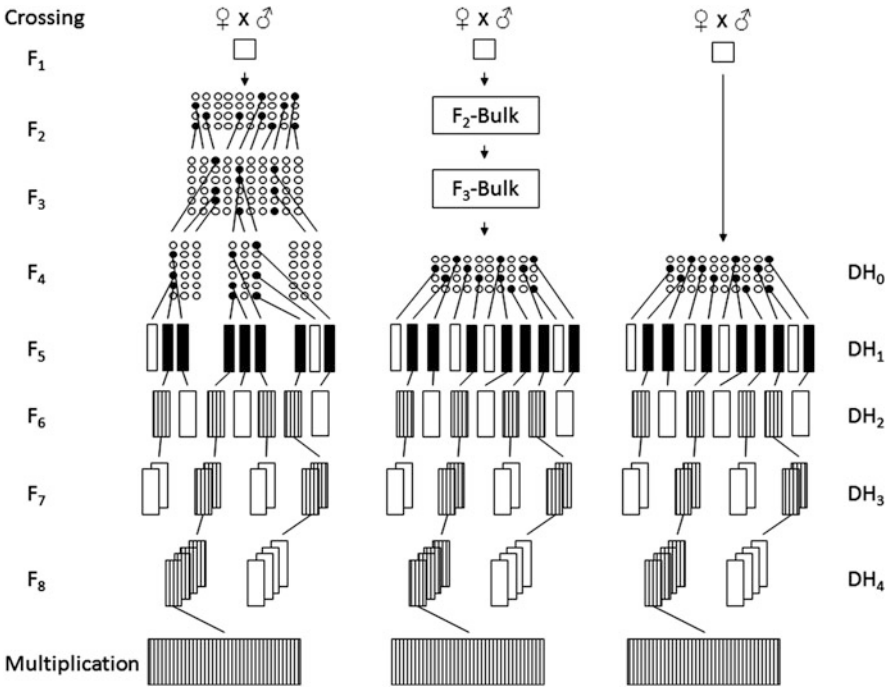

Fig. 1.4 Line breeding examples, with pedigree (*left*), bulk (*middle*), and DH (*right*) selection, modified from Becker (2011)

This principle is repeated again until generation F4 or F5 from which on seed is multiplied in larger batches to allow for larger scale yield testing.

The idea behind pedigree breeding is to judge the potential of a single plant on the basis of the family/progeny performance. Early generation selection is mainly used to select for highly heritable agronomic traits like plant height or lodging, disease resistances, and some quality traits. Pedigree breeding is currently the most widely used method in barley breeding.

1.3.3.3 Bulk Selection

In bulk selection, the whole F2 is harvested and a random sample sown for the next generation. The same applies in F3 and sometimes higher generations. Selection is postponed to a later generation of inbreeding (typically F4) when the plants have reached a higher level of homozygosity and are thus closer to the completely homozygous line which is a new potential variety.

1.3.3.4 Doubled Haploid Selection

The doubled haploid (DH) method can be seen as a variant of the bulk method. Starting from the F1, DH plants are produced (see Sect. 1.3.4) so that full homozygosity is reached in one step. All DH candidates are thus immediately potential pure line varieties, and the breeder "only" has to identify the best DH line. The major advantage of DH selection is a shortening of the breeding scheme. This is especially important in winter barley, where the use of counter-season nurseries to shorten the breeding process is not possible due to the need for vernalization. A disadvantage could be the relative fast loss of genetic variation which could be addressed via pre-breeding activities (Röber et al. 2005).

In all the schemes, once the candidates have reached a sufficient level of homozygosity, all (remaining) candidates are tested in multilocation, multiyear replicated trials in which the focus is clearly on yield and yield stability. Each breeder of course modifies a breeding program to her/his taste and uses opportunities to accelerate with greenhouse and counter-season generations or modern technologies like marker-assisted selection where possible.

Maintenance breeding is started 1 year prior to the final selection year before entering official trials. The process from cross to registration takes at least 8 years, sometimes even 10 or 11.

1.3.3.5 Hybrid Breeding

Although most barley varieties are conventional inbred lines still, a growing potential is seen for hybrid varieties. The main driver has been the breeding company Syngenta who launched the first commercial six-rowed winter hybrid

varieties in 2000 in the United Kingdom. In the last decade, these hybrid varieties have gained a minor but significant share of the market in Germany, United Kingdom, and France (Longin et al. 2012). The hybridization system employed is a cytoplasmic male sterility (CMS) on the seed parent side combined with a dominant restoration of fertility (RF) in the pollinator. Just as in wheat, the level of heterosis (hybrid advantage) achievable in barley is less pronounced than has been experienced in the open pollinating cereals maize and rye. Muehleisen et al. (2013) found an average midparent heterosis of 11.3 % and a commercial heterosis (yield advantage over the best line variety in comparison) of 7.6 %.

One of the problems to overcome in hybrid barley breeding is that barley as a typical self-pollinating crop does not flower openly. In many cultivars, pollination takes place even before anthers are pushed out (Nair et al. 2010). They contain only small amounts of pollen in comparison to, e.g., open pollinating rye. To guarantee sufficient levels of pollination, male hybrid parent lines have to flower more openly, the matching of flowering time between male and female is important, and weather conditions should be favorable during pollination. Still, hybrid seed production remains risky. Nevertheless, depending on further improvements in seed production and development of suitable parent lines, hybrid breeding might provide a major step towards higher barley productivity, as was observed in rice.

1.3.3.6 Pre-breeding

Normally, the word breeding is strongly associated with the development of a new variety, a new product. Pre-breeding describes the area of activities before the product pipeline process (*pre*-phase). Synonyms are trait breeding, scientific breeding, or germplasm enhancement breeding, all directing to the use of genetic resources and the introgression of new alleles/genes into the breeding material.

The classical pre-breeding approach consists of making a (wide) cross between en elite and a donor parent, followed by some rounds of backcrossing to the elite parent and concomitant phenotypic observation if the trait of interest is expressed. The process is tedious and slow and usually only applicable for one trait at a time. Modern approaches like marker-assisted selection (see Sect. 1.3.4) can tremendously speed up this process. The number of backcrosses needed is reduced dramatically, since only those progeny are chosen which carry the gene of interest ("foreground selection") while at the same time having the highest amount of elite genome ("background selection"). Moreover, if there is a reliable trait-marker association, phenotypic evaluation can be reduced to a minimum or skipped totally.

There are many known examples in barley. Jefferies et al. (2003) describe the introgression of the *Yd2* gene providing resistance to barley yellow dwarf virus (BYDV). They used a marker-assisted approach that markedly improved the precision and efficiency of the introgression process.

1.3.4 Novel Breeding Technologies

A number of modern technical innovations have been brought to bear an influence on barley breeding over recent years. Some of these approaches have helped accelerate the breeding process (e.g., microspore culture in the production of DH lines); others have made it more precise and effective (e.g., use of molecular gene markers). Particularly important are those methods which have facilitated early generation and more targeted selection.

1.3.4.1 Doubled Haploid Plants

The production of DH lines is currently a standard method of the creation of new material in most modern barley breeding programs. There are three ways to obtain such lines: (1) by using embryogenic pollen culture, (2) by another culture, and (3) by uniparental genome elimination upon wide crosses (e.g., with *Hordeum bulbosum* as pollinator).

The most advanced and cost-effective method is embryogenic pollen culture in vitro (see Fig. 1.5). In this process, the normal development of immature pollen towards the formation of male gametes is stopped and is diverted to a new developmental pathway involving successive vegetative cell divisions resulting in the formation of callus tissue that ultimately gives rise to haploid plantlets. During this process, two thirds to three quarters of the young plantlets spontaneously double their genome. The result is a "doubled haploid" (DH) plant (Kasha and Kao 1970, see also Chap. 20).

Such DH plants have several advantages: (1) They accelerate the process of getting homozygote lines from four to six generations to merely two. (2) Some major characters like disease resistances can only properly be observed in homozygous state. This enables the possibility for early, small-scale row observations to select on these traits before the candidates enter yield trials. In combination with

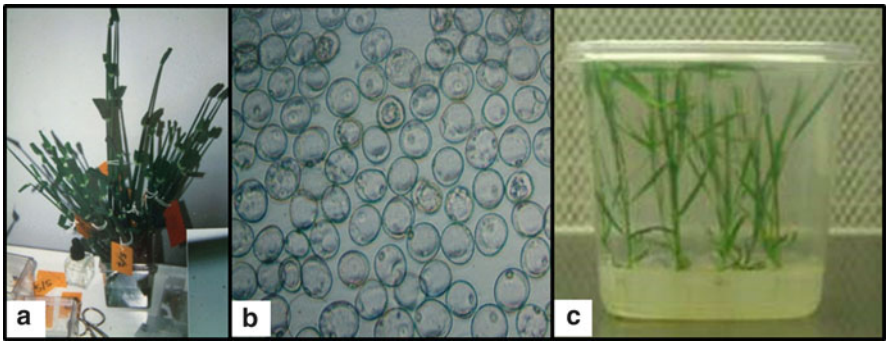

Fig. 1.5 DH development via microspore culture: (**a**) the spikes of barley for microspore isolation; (**b**) barley microspores; and (**c**) green plants regenerated (KWS own images)

marker-assisted selection (MAS, see below), this enables an even more early and robust selection step. (3) Maintenance breeding is much easier and simpler as the starting material is a pure homozygous line.

1.3.4.2 Molecular Markers

With the advent of restriction fragment length polymorphisms (RFLPs), polymerase chain reaction (PCR)-based markers, and particularly with the development of the third generation of DNA markers, single nucleotide polymorphisms (SNPs), it became possible to carry out high-throughput genotyping to map and tag agronomically valuable traits. This approach is applied very actively in today's barley breeding practice. Some examples of such applications are given by Miedaner and Korzun (2012) and explained in details in later chapters of this book. The impact of the technology will doubtlessly continue to grow, as the pace of development in the area of DNA diagnostics is extremely rapid. Saturation of the genetic map with genetic markers and improvements in robotics and high-throughput genotyping are now offering the tantalizing possibility of enhancing major aspects of phenotypic selection with the novel concept of *genomic selection*. The proposed benefit of this technology is that the breeding value of an individual will become largely predictable from its genotype. To support the "mapping" process, precise and robust phenotyping is gaining more importance with the availability of high density marker coverage of the genome. As the genomic marker innovation curve (more throughputs, more density, lower cost, more efficient sampling, etc.) is likely to continue in the near future, the aspect of precise and robust phenotyping could even become a bottleneck (Furbank and Tester 2011).

1.3.4.3 Genetic Engineering

Genetic engineering is very well possible in barley. However, genetically modified varieties are not present in the market due to relatively low trait value, high deregulation cost, low market demand, and a low acceptance in the public, at least in Europe. This constitutes a negative business case.

On the contrary, genetic engineering is regularly used in barley in a research environment, mainly within public institutes, especially for gene-trait evaluation in high-throughput mode. This opens up also the potential to use the abundant variability present in the wild barley relatives since all wild barley species except *H. bulbosum* show crossing barriers to cultivated barley. Examples of beneficial targets are disease and pest resistances as well as tolerances to abiotic stresses like cold, salt, and drought. In the end, the results of such gene-trait studies could either lead to genetically modified varieties, if there is a business case, or be used in "classical," nongenetically engineered barley breeding employing modern technologies like targeted mutation breeding.

Conclusion

Barley is the fourth important cereal crop in the world with major usage in feed for cattle and pigs as well as in the malting industry for beer or spirit production. We see a relatively stable situation in both the production and the usage in foreseeable future, enabling research and breeding to continue improvements of the crop. We assume this is true for the more productive winter barleys in the moderate maritime climate, as well as for spring barleys with major growth areas in continental and subtropical climates.

At present, a modern barley breeding program is a combination of classical breeding and novel technologies like molecular marker application and DH production. Incorporation of novel technologies in the barley breeding process has markedly accelerated the time from initial cross to variety release while maintaining an average yield increase and improving the resistance and quality levels. For the future, yield, resistances, and quality characteristics remain the important fields to be worked on to ensure the success of barley.

A positive development is the rapid accumulation of genomic tools in cereal crops observed during the last decade, which was mainly led by barley as a model crop. Together with the availability of new "tools and technologies," from hybrid genetic systems to genomic selection, we believe barley breeding should be able to keep pace with the rapid developments in the three major staple crops: wheat, maize, and rice. Combined with the agronomic potential of the barley crop, it is most likely that barley will hold its place in crop rotations and maintain its importance in the feed and food value chain.

References

Badr A, Müller K, Schäfer-Pregl R, El Rabey H, Effgen S, Ibrahim HH, Pozzi C, Rohde W, Salamini F (1999) On the origin and domestication history of barley (*Hordeum vulgare*). Mol Biol Evol 17:499–510

Becker HC (2011) Pflanzenzüchtung, 2nd edition (updated). Eugen Ulmer Verlag, Stuttgart. ISBN 978-3-8252-3558-1

Bowman JGP, Blake TK, Surber LMM, Habernicht DK, Bockelman H (2011) Feed-quality variation in the barley core collection of the USDA national small grains collection. Crop Sci 41:863–870

Comadran RL, Thomas WTB, Macaulay M, Marshall DF, Shaw P, Cockram J, White J, Mackay I, Liu Z, Kearsey MJ, O'Sullivan D, Waugh R (2010) Association genetic analysis of UK elite barley platform. Monogram Network Workshop, Norwich, UK, 2–4 March 2010

Eden KJ (Trans) (1993) History of German brewing. Zymurgy Mag. 16.4 Special 1993. www.Brewer.org. Accessed 26 Oct 2011

FAOSTAT (2011) Food and agriculture organization of the United Nations. http://faostat.fao.org. © FAO Statistics Division

Furbank RT, Tester M (2011) Phenomics – technologies to relieve the phenotyping bottleneck. Trends Plant Sci 16:635–644

Harrold RL, Dinusson WE, Haugse CN, Johnson JN (1971) Barley in rations for early-weaned pigs. North Dakota Farm Res 28(6):3

Herz M (2012) LfL Freising. Markus.Herz@lfl.bayern.de, Germany (personal conveyance)
International Grains Council (2012) Five-year global supply and demand projections to 2017/18. http://www.igc.int/en/downloads/grainsupdate/IGC_5year_projections.pdf. International Grains Council
Jefferies SP, King BJ, Barr AR, Warner P, Logue SJ, Langridge P (2003) Marker-assisted backcross introgression of the *Yd2* gene conferring resistance to barley yellow dwarf virus in barley. Plant Breed 122:52–56
EFSA Journal (2011) Scientific opinion on the substantiation of a health claim related to barley beta-glucans and lowering of blood cholesterol and reduced risk of (coronary) heart disease pursuant to Article 14 of Regulation (EC), No 1924/2006. http://www.efsa.europa.eu/en/efsajournal/doc/2471.pdf, 9(12):2471
Kasha KJ, Kao KN (1970) High frequency haploid production in Barley (*Hordeum vulgare* L.). Nature 225:847–875
Kirchgeßner M, Roth FX, Schwarz FJ, Stangl GI (2011) Tierernährung. DLG. ISBN 978-3-7690-0803-6
Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H et al (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci U S A 104:1424–1429
Kunze W (2010) Technology brewing and malting, Chap 4, 4th updated edn. VLB, Berlin, p 609
Longin CFH, Muehleisen J, Maurer HP, Zhang H, Gowda M, Reif JC (2012) Hybrid breeding in autogamous cereals. Theor Appl Genet 125:1087–1096
Lüpken T et al (2013) Genomics-based high-resolution mapping of the BaMMV/BaYMV resistance gene rym11 in barley (Hordeum vulgare L.). Theor Appl Genet 126:1201–1212
McCouch S et al (2013) Feeding the future. Nature 499:23–24
Michel RH, McGovern PE, Badier VR (1993) The first wine and beer: chemical detection of ancient fermented. Anal Chem 65:408A–413A
Miedaner T, Korzun V (2012) Marker-assisted selection for disease resistance in wheat and barley breeding. J Phytopathol 102:560–566
Muehleisen J, Maurer HP, Stiewe G, Bury P, Reif JC (2013) Hybrid breeding in barley. Crop Sci 53:819–824
Nair SK et al (2010) Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. Proc Natl Acad Sci U S A 107:490–495
Nilan RA, Ullrich SE (1993) Barley origin, taxonomy, distribution, genetics and breeding. In: MacGregor AW, Bhatty RS (eds) Barley: chemistry and technology, Chap 1. American Association for Cereal Chemists, St. Paul, MN, pp 1–29
Olsen JE, Bindi M (2002) Consequences of climate change for European agricultural productivity, land use and policy. Eur J Agron 16:239–262
Reid A (2009) Economic report on Scottish agriculture. ISBN 978-0-7559-8058
Röber FK, Gordillo GA, Geiger HH (2005) In vivo haploid induction in maize – performance of new inducers and significance of doubled haploid lines in hybrid breeding. Maydica 50:275–283
Scholz M (2009) Ryd4Hb: a novel resistance gene introgressed from Hordeum bulbosum into barley and conferring complete and dominant resistance to the barley yellow dwarf virus. Theor Appl Genet 119:837–849
Setotaw TA, Daba SD, Belayneh SG, Al-Yassin A (2012) ICARDA and EIAR success of joint barley-breeding program in developing food barley varieties for the Ethiopian low-moisture areas. Int J Water Resour Arid Environ 2(1):24–30 (ISSN 2079-7079)
Von Zitzewitz J, Cuesta-Marcos A, Condon F, Castro A, Chao S, Corey A, Filichkin T, Fisk SP, Gutierrez L, Haggard K, Karsai I, Muehlbauer G, Smith K, Veisz K, Hayes P (2011) The genetics of winter hardiness in barley: perspectives from association mapping. Plant Genome 4:76–91
Ziegler K (2012) Nutrition and management: characteristics of common feed grains. http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/beef11489, cited 25 Feb 2007, reviewed 12 Oct 2012

Chapter 2
Genetic Diversity and Germplasm Management: Wild Barley, Landraces, Breeding Materials

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2.1 Introduction

The ancestral form of cultivated barley, *Hordeum vulgare* ssp. *spontaneum*, is a source of diversity for its cultivated form *Hordeum vulgare* ssp. *vulgare*. The evolution of this wild plant in the Near East has resulted in a complex biological specialisation across the species range, which is associated with a large genetic diversity. The domestication process narrowed the diversity of the early cultivated forms, introducing a genetic 'bottleneck', even though introgression from the wild form can and does still occur. After the selection of the important domestication mutation of brittle rachis (shattering), early farmers selected agronomically important mutants, such as six-rowed, spring habit or hulless types within a few thousand years. The geographical distribution of cultivated barley diversity is significantly

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correlated with genes of adaptation to their ecological conditions and to different uses, e.g. for food, feed and malt. These naturally occurring polymorphisms were the only sources of diversity to early farmers and were available as haplotypes in landraces until the cross-breeding activity of barley started in the early twentieth century (Fischbeck 2003). These natural and artificial diversities of the *H. vulgare* gene pool are preserved mostly in the form of genebank collections which are the main topics in this chapter.

2.2 Taxonomy of the Genus *Hordeum*

The genus *Hordeum* belongs to the tribe Triticeae of the family Poaceae (Gramineae). The tribe includes a number of important cereal crops, such as wheat (*Triticum* spp.), rye (*Secale cereale*), barley (*Hordeum vulgare*) and artificially developed triticale. In addition to these cereals, many important forage grass species are referred to this tribe. In the genus *Hordeum*, von Bothmer and Jacobsen (1985) recognised four sections: section *Hordeum*, section *Anisolepis*, section *Stenostachys* and section *Critesion*. Blattner (2009) further proposed a finer structure of infrageneric categories using a multitude of loci from the chloroplast and nuclear genomes. The taxa of *Hordeum* are described in Table 2.1 by the analysis of Blattner (2009). Detailed description of the species in *Hordeum* is given in von Bothmer et al. (1995).

2.3 Gene Pools of Barley

Barley germplasm can be divided into major groups, namely, (1) cultivars, (2) landraces, (3) breeding lines, (4) wild *Hordeum* species and (5) genetic stocks. An alternative classification of primary, secondary and tertiary gene pools has also been used (von Bothmer et al. 2003). The primary gene pool consists of cultivated barley and wild *H. vulgare* ssp. *spontaneum*. Gene transfer into the barley crop by crossing is easy within this gene pool. The secondary gene pool includes related *Hordeum* species whose gene transfer to the crop is possible but difficult in practice. The description of the barley gene pools and the taxonomic concept of the genus *Hordeum* are based on von Bothmer et al. (1995). When applied to cultivated barley and its wild relatives, the gene pool concept presents a very clear-cut picture as shown in von Bothmer et al. (2003).

The barley primary gene pool includes cultivars, landraces and breeding lines together with the wild ancestral form of domesticated barley, *H. vulgare* ssp. *spontaneum*. Crossing combinations of cultivated barley with this form show no incompatibility barriers; hence, there is a full capacity for gene transfer. This gene pool includes main germplasm in current breeding activities. Landraces are still cultivated in Asia and North Africa including Ethiopia and have been used until

Table 2.1 Taxa of the genus *Hordeum* (Blattner 2009)

Taxon	Ploidy	Haploid genome	Distribution area
Subgenus *Hordeum*			
Section *Hordeum*			
H. vulgare L.			
subsp. *vulgare*	2×	H	Cultivated
subsp. *spontaneum* (C. Koch.) Thell.	2×	H	SW Asia
H. bulbosum L.	2×, 4×	H, HH	Mediterranean to C Asia
Section *Trichostachys* Dum.			
H. murinum L.			
subsp. *glaucum* (Steud.) Tzvel.	2×	Xu	Mediterranean to C Asia
subsp. *murinum*	4×	XuXu	NW Europe to Caucasus
subsp. *leporinum* (Link) Arc.	4×, 6×	XuXu, XuXuXu	Mediterranean to C Asia
Subgenus *Hordeastrum* (Doell) Rouy			
Section *Marina* (Nevski) Jaaska			
H. gussoneanum Parl.	2×, 4×	Xa, XaXa	Mediterranean to C Asia
H. marinum Huds.	2×	Xa	Mediterranean
Section *Stenostachys* Nevski			
Series *Sibirica* Nevski			
H. bogdanii Will.	2×	I	C Asia
H. brevisubulatum (Trin.) Link[a]	2×, 4×, 6×	I, II, III	C Asia
H. roshevitzii Bowden	2×	I	C Asia
Series *Critesion* (Raf.) Blattner comb. & stat. nov.			
H. californicum Covas & Stebb.	2×	I	SW USA
H. chilense Roem. & Schult.	2×	I	Chile and W Argentina
H. comosum Presl	2×	I	S Argentina
H. cordobense Bothmer et al.	2×	I	C Argentina
H. erectifolium Bothmer et al.	2×	I	C Argentina
H. euclaston Steud.	2×	I	C Argentina, Uruguay
H. flexuosum Steud.	2×	I	E + C Argentina
H. intercendens Nevski	2×	I	SW USA, NW Mexico
H. muticum Presl	2×	I	C to N Andes
H. patagonicum (Haum.) Covas[a]	2×	I	S Argentina
H. pubiflorum Hook. f.[a]	2×	I	S Argentina
H. pusillum Nutt.	2×	I	C + E USA
H. stenostachys Godr.	2×	I	C Argentina
H. depressum (Scribn. & Sm.) Rydb.	4×	II	W USA

(continued)

Table 2.1 (continued)

Taxon	Ploidy	Haploid genome	Distribution area
Interserial allopolyploids of series *Critesion* (all combining genomes of an American species with most probably one derived from *H. roshevitzii*)			
H. brachyantherum Nevski	4×	II	W North America, Kamchatka, Newfoundland
H. fuegianum Bothmer et al.	4×	II	S Argentina, S Chile
H. guatemalense Bothmer et al.	4×	II	Guatemala, S Mexico
H. jubatum L.	4×	II	NE Asia, NW + W North America
H. tetraploidum Covas	4×	II	C Argentina
H. arizonicum Covas	6×	III	SW USA
H. lechleri (Steud.) Schenk	6×	III	C + S Argentina
H. parodii Covas	6×	III	C Argentina
H. procerum Nevski	6×	III	S Argentina
Section *Nodosa* (Nevski) Blattner comb. & stat. nov.			
H. brachyantherum Nevski	6×	IIXa	C California
H. capense Thunb.	4×	IXa	S Africa
H. secalinum Schreb.	4×	IXa	Mediterranean, C Europe

Detailed description of the species in *Hordeum* is given in von Bothmer et al. (1995)
[a]Species with subspecies not further detailed here

recently in other areas. *H. vulgare* ssp. *spontaneum* has been used for transfer of disease resistance genes into barley germplasm (Fischbeck 2003). In recent years, molecular marker studies identified in *H. vulgare* ssp. *spontaneum* a number of diverse genes of potential value for barley improvement such as recombinant chromosome substitution lines (Pillen et al. 2003, 2004; Matus et al. 2003; Sato and Takeda 2009; Inostroza et al. 2009).

The secondary gene pool includes only a single species, *H. bulbosum* (bulbous barley), sharing the H genome with barley (Blattner 2009), which crosses with some difficulty (needing embryo rescue sometimes). However, in recent years, it has been demonstrated that genes from *H. bulbosum* can be transferred to cultivated barley, thus providing a new source for breeding (Pickering 2000). The diploid *H. bulbosum* was used for the production of doubled haploids in barley breeding through chromosome elimination (Kasha and Kao 1970; Pickering 1984; Chen and Hayes 1989).

The tertiary barley gene pool includes all the remaining species of *Hordeum*. Crossing with *H. vulgare* is difficult and backcrossing to the crop is almost impossible (von Bothmer et al. 1983; von Bothmer and Linde-Laursen 1989). The potential for barley improvement from this gene pool is therefore very limited, unless advanced techniques, such as somatic hybridisation and transformation, can be applied.

2.4 Barley *Ex Situ* Collections

The FAO (1996) estimated that about 485,000 barley accessions exist in *ex situ* germplasm collections. van Hintum and Menting (2003) were able to estimate the number of duplicates and revised this figure to ca. 371,000 accessions. Similarly, the Global Crop Diversity Trust (2008) estimated the number of barley accessions in genebanks worldwide to be 370,796. In 2010 'The second report on the on the State of the World's Plant Genetic Resources for Food and Agriculture' was published by FAO (2010) and subsequently listed 466,531 barley accessions.

2.4.1 Size and Composition of Collections

Table 2.2 lists the major barley collections (FAO 2010). This total is 4 % lower than the FAO's estimate in 1996 and 26 % higher than more recently reported by van Hintum and Menting (2003) and Global Crop Diversity Trust (2008).

Wild barleys: A major part of the wild relative collections is represented by *H. vulgare* ssp. *spontaneum*. According to Global Crop Diversity Trust (2008), 34,279 accessions are listed in major collections. Main collections are maintained in several institutions in the UK, Israel, PGRC, NSGC, IPK and at ICARDA. The *spontaneum* collection held at ICARDA is derived from 730 original populations that originate from 20 countries and is considered as globally most diverse source ecologically (Global Crop Diversity Trust 2008). A large number of accessions are held at IPSR (John Innes Centre), UK but is a selection from a limited number of sites, rather than a geographically representative collection. Main collections of other wild *Hordeum* species of the secondary and tertiary gene pools are held at PGRC and NORDGEN.

Landraces: Landraces represent the largest part of barley germplasm in genebanks (23 %). The greatest number of landraces is held in five genebanks: ICARDA, IBC, PGRC, NSGC and IPK, each having more than 10,000 accessions.

Breeding lines: A large number of breeding lines are also held in genebanks, mainly at CIMMYT, NORDGEN, PGRC, NSGC, IHAR and IPSR with more than 3,000 accessions.

Advanced cultivars: This category includes released cultivars from breeding programmes. The large cultivar collections are maintained by PGRC, NSGC, IPK and IPSR.

Genetic stocks: The most extensive collection of genetic stock materials is held at NORDGEN. This collection comprises about 10,000 accessions from joint Scandinavian mutation research programmes and 685 translocation and 58 duplication lines (Global Crop Diversity Trust 2008). Large collections of genetic stocks are also maintained at PGRC, NSGC and NIAR.

Table 2.2 Collections of barley germplasm accessions

Country	Genebank		Accessions	
	Code	Name	No.	%
Canada	PGRC	Plant Gene Resources of Canada, Saskatoon Research Centre, Agriculture and Agri-Food Canada	40,031	8.6
USA	NSGC	National Small Grains Germplasm Research Facility, United States Department of Agriculture, Agricultural Research Services	29,874	6.4
Brazil	CENARGEN	Embrapa Recursos Genéticos e Biotecnologia	29,227	6.3
Global	ICARDA	International Centre for Agricultural Research in the Dry Areas	26,679	5.7
Japan	NIAS	National Institute of Agrobiological Sciences	23,471	5.0
Germany	IPK	Leibniz Institute of Plant Genetics and Crop Plant Research	22,093	4.7
China	ICGR-CAAS	Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences	18,617	4.0
Korea	RDAGB-GRD	Genetic Resources Division, National Institute of Agricultural Biotechnology, Rural Development Administration	17,660	3.8
Russia	VIR	N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry	16,791	3.6
Ethiopia	IBC	Institute of Biodiversity Conservation	16,388	3.5
Mexico	CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trig	15,473	3.3
Sweden	NORDGEN	Nordic Genetic Resources Centre	14,109	3.0
UK	IPSR	Department of Applied Genetics, John Innes Centre	10,838	2.3
India	NBPGR	National Bureau of Plant Genetic Resources	9,161	2.0
Australia	SPB-UWA	School of Plant Biology, Faculty of Natural and Agricultural Sciences, University of Western Australia	9,031	1.9
Iran	NPGBI-SPII	National Plant Gene Bank of Iran, Seed and Plant Improvement Institute	7,816	1.7
Israel	ICCI-TELAVUN	Lieberman Germplasm Bank, Institute for Cereal Crops Improvement, Tel-Aviv University	6,658	1.4
Poland	IHAR	Plant Breeding and Acclimatization Institute	6,184	1.3
Bulgaria	IPGR	Institute for Plant Genetic Resources	6,171	1.3
Others (180)			140,259	30.1
Total			466,531	100.0

The collections are listed by institutions in descending order of the collection size

2.4.2 Storage Duplication

The largest collection of barley germplasm in Table 2.2 is held by Plant Gene Resources of Canada (PGRC) which includes a large duplicated USDA collection.

According to van Hintum and Menting (2003) and Global Crop Diversity Trust (2008), considerable duplications exist between the four globally largest collections, i.e. PGRC, USGC, CENARGEN and ICARDA, particularly in the category of cultivars. These duplications themselves have functions of safety duplications. Also, some genebanks have own safety duplications in their organisations. An analysis of the data in the Global Inventory of Barley Genetic Resources (Global Crop Diversity Trust 2008) indicates that the highest number of duplicates is among the cultivars—43,000 accessions to 8,850 cultivars—whereas in the total of 50,000 landrace accessions, 61 % may be unique, and among 23,700 breeding lines, the proportion of unique accessions is even higher (71 %). To duplicate all unique accessions, the following points were suggested in the discussion of Tunis meeting organised by the Global Crop Diversity Trust (Global Crop Diversity Trust 2008):

- A proposed definition of safety duplication was a formal agreement in long-term storage in distant location not necessarily as black box.
- First priority should be given to most valuable unique diversity of wild relatives, landraces and genetic stocks, second priority to cultivars and a third priority to breeding material.
- It was proposed by the group to look into the information on safety duplication in the global barley registry.
- ICARDA facilities were suggested as the most appropriate location for hosting safety duplicates, but due to difficult political conditions is inaccessible.
- In addition to safety duplication under long-term storage conditions in a genebank, a second level of safety duplication is highly desirable, such as the Svalbard Global Seed Vault facilities for deep freeze storage. In September 2014, approximately 70,000 *Hordeum* accessions (68,825 are ssp. *vulgare*) were stored there (http://www.nordgen.org/sgsv/).

2.4.3 Core Collection

To improve the accessibility of large collections as described in Table 2.2, the concept of core collections was developed. The International Barley Core Collection (BCC) is a selected and limited set of accessions developed in 1989 by an international consortium as an activity of the participating institutions (Knüpffer and van Hintum 2003). It includes a set of barley genotypes for research and represents the genetic diversity of cultivated and wild species of *Hordeum*, covering the three gene pools and including well-known genetic stocks.

The accessions of BCC were selected by subset coordinators below (Global Crop Diversity Trust 2008), via single seed descent. The institutions of subset coordinators are also responsible for initial multiplication of accessions and distribution of BCC samples to users in their respective regions. At present, the size of the BCC does not exceed 1,500 accessions. The accessions are divided in the following subsets.

1. Landraces and cultivars from West Asia and North Africa (WANA): The selection of 285 accessions of the WANA subset was initiated at ICARDA.
2. Landraces and cultivars from South and East Asia: Okayama University, Japan, is responsible for selecting and managing 380 East Asian accessions from Japan, Korea, China, Nepal, Bhutan and India.
3. Landraces and cultivars from Europe: An initial set of 320 European barley landraces and cultivars was selected by IPK, Germany.
4. Landraces and cultivars from the Americas: A selection of 155 accessions was made by NSGC, USA, in the materials of the USA, Canada, Mexico and several South American countries.
5. Cultivars from Oceania and other parts of the world: A selection of ten Australian and one New Zealand cultivars has been made by AWCC, Australia.
6. *Hordeum vulgare* ssp. *spontaneum*: A set selected by ICARDA includes 150 accessions from 17 countries.
7. Other wild *Hordeum* species: Two entries were selected from each species (when available). The cross-pollinating species *H. bulbosum* and *H. brevisubulatum* are not yet available, but the other 45 entries of 22 species are available from NORDGEN.
8. Genetic stocks: The selection and preparation of the subset with genetic stock was completed. Most of the material is available from NSGC, USA.

2.4.4 Information and Data Management

Table 2.3 summarises Internet accesses of databases on the status of collections regarding passport and characterisation/evaluation data (Global Crop Diversity Trust 2008). Passport information is available in most of the databases; however, availability of characterisation/evaluation data is poor and only some are searchable. Specialised databases have been developed to link different sources of data, such as CGIAR System-wide Information Network for Genetic Resources (SINGER http://singer.cgiar.org/), European PGR catalogue (EURISCO: http://eurisco.ecpgr.org/) and the European Barley Database (EBDB: http://barley.ipk-gatersleben.de/ebdb.php3). The GENESYS project (http://www.genesys-pgr.org/) tries to connect and simplify these information systems. It is an important and rich source of information on plant genetic resources, diversity of seeds conserved in genebanks worldwide and crops and crop-wild relative material for use by researchers, students, breeders, farmers and decision-makers.

The databasing of marker-associated genetic diversity has lagged behind the descriptive databases surveyed above, largely because new database structures are required to accommodate and process the huge and exponentially increasing volumes of data becoming available. An example of one of the first databases to include marker data is the GERMINATE website, which is used worldwide for many crops including barley (Lee et al. 2005; http://bioinf.scri.ac.uk/public/?page_id=159).

Table 2.3 Barley collection databases (Global Crop Diversity Trust 2008)

Country	Genebank	URL (Cited 28 Feb 2013)
Canada	PGRC	http://pgrc3.agr.ca/search_grinca-recherche_rirgc_e.html
USA	NSGC	http://www.ars-grin.gov/npgs
Global	ICARDA	http://singer.cgiar.org/
United Kingdom	IPSR	http://www.jic.ac.uk/GERMPLAS/bbsrc_ce/index.htm
Germany	IPK	http://gbis.ipk-gatersleben.de
China	ICGR-CAAS	http://icgr.caas.net.cn/cgris_english.html
Russia	VIR	http://www.vir.nw.ru/data/dbf.htm
Japan	Okayama U	www.shigen.nig.ac.jp/barley/
Sweden	NORDGEN	www.nordgen.org/ngb/ (Latvia and Estonia included)
Australia	AWCC	http://www2.dpi.qld.gov.au/extra/asp/auspgris/
Global	CIMMYT	http://singer.cgiar.org/
Japan	NIAS	http://www.gene.affrc.go.jp/plant/
Israel	ICCI-TELAVUN	http://www2.tau.ac.il/ICCI/default.asp
Bulgaria	IPGR	http://eurisco.ecpgr.org
Netherlands	GGN	http://www.cgn.wur.nl/UK/
Israel	IGB	http://igb.agri.gov.il/
Czech Republic	RICP	http://genbank.vurv.cz/genetic/resources/
Slovakia	SVKPIEST	http://eurisco.ecpgr.org
Romania	BRGV	http://www.svgenebank.ro/index.htm

See Table 2.2 for genebank codes other than indicated: IPSR = Department of Applied Genetics, John Innes Centre, Norwich Research Park; AWCC = Australian Winter Cereals Collection; CGN = Centre for Gentic Resources; IGB = Israel Gene Bank for Agricultural Crops, Agricultural Research Organization, Volcani Centre; SVKPIEST = Research Institute of Plant Production Piestany; BRGV = Suceava Genebank

2.4.5 Distribution

Table 2.4 presents a list of barley collections most active in germplasm distribution (Global Crop Diversity Trust 2008). International distribution is limited even in genebanks with large number of collections. This may be a reason to have large sets of duplicated accessions. USDA, ICARDA and IPK are major providers with more than 2,000 accessions sent out yearly.

2.5 Management of Diversity

With the recent advances of genome-related technologies, e.g. genome-wide single nucleotide polymorphisms (SNPs), genotyping genebank accessions including exotic germplasm will be more accessible and cost effective. Genebank collections

Table 2.4 Annual barley accession distribution (Global Crop Diversity Trust 2008)

Country	Institute	Distributed annually		
		Nationally	Internationally	Total
USA	NSGC	5,000	3,000	8,000
Global	ICARDA	2,800 (to ICARDA)	2,600	5,400
Germany	IPK	1,731	2,326	4,057
Australia	AWCC	3,256	164	3,420
Russia	VIR	2,958	350	3,308
Japan	Okayama U	1,252	387	1,639
Czech R.	ARI Kromeriz	1,210	256	1,466
Sweden	NORDGEN	250 (to Nordic countries)	500	750

can be managed more appropriately with DNA markers for accession identification, estimates of diversity and trait association. A genome-wide DNA marker system also provides a means to monitor and transfer genes or segments in exotic germplasm to evaluate their performances under the well-studied genetic background as demonstrated by the development of introgression lines.

2.5.1 Genetic Diversity Within Collections

Microsatellite markers or simple sequence repeats (SSRs) from anonymous (g-SSRs) and genic regions of the genome (e-SSRs) have been extensively used for genetic diversity studies in barley (Saghai-Maroof et al. 1994; Becker and Heun 1995; Russell et al. 1997, 2003, 2004; Struss and Plieske 1998; Pillen et al. 2000; Kota et al. 2001a, b; Matus and Hayes 2002; Ivandic et al. 2002; Koebner et al. 2003; Thiel et al. 2003; Malysheva-Otto et al. 2006; Kolodinska et al. 2007; Varshney et al. 2007; Yahiaoui et al. 2008; Naeem et al. 2011).

The earlier studies served to establish SSRs as the method of choice for diversity analysis among individuals, using only a few SSR loci to distinguish between closely related accessions (Saghai-Maroof et al. 1994; Becker and Heun 1995; Russell et al. 1997). Initially several authors used SSRs to examine diversity within the cultivated gene pool cataloguing the changes that have arisen since modern breeding began in the early 1900s. Russell et al. (2000), using 28 mapped SSRs, observed an overall reduction in diversity over time and highlighted chromosomal regions with limited diversity. Although Kolodinska et al. (2007), examining changes in diversity among 197 Nordic barleys, did not observe a decrease of diversity over time, they did observed differences between Northern and Southern germplasm. In contrast, Koebner et al. (2003) and Malysheva-Otto et al. (2007) concluded that systematic barley breeding in the twentieth century had not resulted in significant reduction of genetic diversity. Despite the differences in results, all authors recognised that SSR diversity was a better measure of genetic distance than botanical or kinship measures (Matus and Hayes 2002).

As well as comparing early and modern cultivars, many authors have categorised the differences between cultivars, landraces and wild barleys using SSRs. The main conclusion from all of these studies is that allelic diversity is greater in wild than in cultivated barley. These early studies were constrained by the time consuming and costly approaches used to develop SSRs (small insert library construction, hybridisation with SSR-repeat oligonucleotides and subsequent sequencing of candidate clones), but this all changed with advances in sequencing technologies as we entered the genomics era. Many barley researchers began to develop expressed sequence tag (EST) libraries which could be mined for microsatellites, and much larger numbers of markers became available for genotyping large collections of accessions. The first studies used only a few accessions to test large numbers of EST-SSRs or e-SSRs (Pillen et al. 2000; Kota et al. 2001a, b; Thiel et al. 2003), and several studies compared the use of genomic SSRs and genic SSRs to address questions relating to diversity and discrimination (Russell et al. 2003; Khlestkina et al. 2006; Varshney et al. 2007).

With these new developments, germplasm collections were being evaluated at the molecular level. The Spanish core collection of 225 accessions, representing over 2,000 Mediterranean barleys, was genotyped using 64 genic SSRs, highlighting the uniqueness and value of these accessions for barley improvement (Yahiaoui et al. 2008). Similar studies have been conducted to examine the diversity in WANA barley collections using SSRs. Orabi et al. (2007) observed the complex genetic structure and close relationships between landraces and wild barleys from WANA region collections. A subset of the collection held at ICARDA representing landraces and wild barleys from 30 countries was recently genotyped with SSRs, highlighting the considerable variation with these collections (Varshney et al. 2010).

As well as gene-based SSRs, the use of SNPs is gaining momentum for understanding genetic diversity in germplasm collections. The international barley community developed a high-throughput SNP genotyping platform based on the Illumina GoldenGate and Oligo Pool Assays (OPA) (Rostoks et al. 2005; Close et al. 2009). These BOPA Illumina SNP markers have been applied in multiple applications including estimation of the genetic variation within national and international collections of cultivated, landrace and wild barley. Although, caution must be exercised when using this marker set to study wider germplasm because ascertainment bias (see Chap. 18) distorts the diversity revealed in landrace and wild germplasm (Moragues et al. 2010); nevertheless, application of the BOPA1 SNP set to wild and landrace barley germplasm from the Middle East has shown that landrace and wild barley populations are genetically differentiated, although clear evidence exists for gene flow from cultivated germplasm into the wild (Russell et al. 2011; Hübner et al. 2012). In addition, significant fluctuations in diversity are seen between wild and landrace barleys in the vicinity of genes known to be involved in barley domestication. The leakage of alleles from cultivated to wild gene pools needs to be taken into account when considering wild barley diversity.

As molecular marker technology advances and become more cost effective, it is envisaged that it will be feasible in the coming years to genotype entire barley

germplasm collections. A sensible option available currently is Illumina SNPs using a BeadXpress 384 SNP set (Moragues et al. 2010) and it is likely that genotyping by sequencing (Elshire et al. 2011) will become available in the near future, raising genomic resolution by more than an order of magnitude.

2.5.2 Introgression Lines

Since the ancestral form of cultivated barley does not have any recombination barriers, it has been used in breeding to bring new sources of genetic variation. However, a biparental cross may bring too many negative traits in wild plants which cannot be removed from usual hybrid breeding programme. Historically, backcrossing has used to remove deleterious alleles following introduction of simple Mendelian segregation traits, e.g. disease resistance (Fischbeck 2003). The idea of backcross introgression populations having every segment of wild genome in the background of cultivated barley (also called recombinant chromosome substitution lines; RCSLs) provides the opportunity to assess unadapted alleles in an adapted genetic background (Pillen et al. 2003, 2004; von Korff et al. 2004; Matus et al. 2003; Schmalenbach et al. 2008; Sato and Takeda 2009). This is not an applicable technique to the secondary or tertiary gene pools of barley; however, it provides the chance of evaluation in never treated traits, e.g. seed productivity in wild barley germplasm.

Sato and Takeda (2009) demonstrated systematic generation of substituted segments of the ssp. *spontaneum* accession H602 into cultivated Haruna Nijo. They also used this system to separate seed dormancy QTLs to simplify genetic analyses on individual single loci (Sato et al. 2009). The same wild barley accession was substituted into the North American elite malting barley cultivar Harrington to find QTLs for multiple disease resistance phenotypes (Yun et al. 2006) and morphological traits (Gyenis et al. 2007).

Recently, a nested association mapping population, comprising nearly 1,500 recombinant lines derived from crosses between 25 diverse ssp. *spontaneum* lines and the cultivar Barke, has been developed (Klaus Pillen, personal communication). This population offers the opportunity to trial introgressed segments of multiple allelic variants from wild germplasm in a common cultivar background.

The necessary technique to identify chromosomal substitution is high-density gene-based marker maps. As mentioned above, a large number of expressed sequence tags (ESTs) have been obtained and used to design single nucleotide polymorphism (SNP) assays for mapping (e.g. Rostoks et al. 2005; Close et al. 2009). This Illumina GoldenGate SNP detection provides the high-throughput parallel detection system of substitution segment of RCSLs as demonstrated in Sato and Takeda (2009).

The idea of an introgression population is not limited to the combination of wild and cultivated barleys but also between cultivated barleys. Sato et al. (2011) developed RCSLs of food barley Akashinriki in the background of malting barley

'Haruna Nijo' to bring exotic alleles in malting barley germplasm. The development of introgression lines also accelerate the isolation of quantitatively inherited trait as demonstrated in rice (Fukuoka et al. 2009) and in barley (Sato and Takeda 2009).

Conclusions
To continue improvements in breeding new cultivars, the diversity locked away in large germplasm collections must be utilised. The accessibility of genebank information has been greatly improved by the Internet web browsing system, although the exchange of seed has become more strictly regulated. We must solve these political problems and prepare for the further use of current barley diversity. Techniques based on genome sequences and resulting tools will accelerate the precise use of current diversity preserved in genebank collections.

References

Becker J, Heun M (1995) Barley microsatellites:allele variation and mapping. Plant Mol Biol 27:835–845

Blattner FR (2009) Progress in phylogenetic analysis and a new infragenetic classification of barley genus *Hordeum* (Poaceae:Triticeae). Breed Sci 59:471–480

Chen F, Hayes PM (1989) A comparison of *Hordeum bulbosum* mediated haploid production efficiency in barley, using in vitro floret and tiller culture. Theor Appl Genet 77:701–704

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney R, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:82

Elshire R, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 6: e19379

FAO (1996) FAO State of the World's Plant Genetic Resources for Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome

FAO (2010) The second report on the state of the world's plant genetic resources for food and agriculture. Commission on genetic resources for food and agriculture, food and agriculture organization of the United Nations, Rome, 399 pp. http://www.fao.org/docrep/013/i1500e/i1500e00.htm. Cited 28 Feb 2013

Fischbeck G (2003) Diversification through breeding. In: von Bothmer R, van Hintum T, Knüpffer H, Sato K (eds) Diversity in barley (*Hordeum vulgare*). Elsevier Science B.V., Amsterdam, pp 29–52

Fukuoka S, Saka N, Koga H, Ono K, Shimizu T, Ebana K, Hayashi N, Takahashi A, Hirochika H, Okuno K, Yano M (2009) Loss of function of a proline-containing protein confers durable disease resistance in rice. Science 325:998–1001

Global Crop Diversity Trust (2008) Priority crops – barley. http://www.croptrust.org/main/priority.php?itemid=148. Cited 28 Feb 2013

Gyenis L, Yun SJ, Smith KP, Steffenson BJ, Bossolini E, Muehlbauer GJ (2007) Genetic architecture of quantitative trait loci associated with morphological and agronomic trait differences in a wild by cultivated barley cross. Genome 50:714–723

Hübner S, Flavell AJ, Fridman E, Graner A, Korol A, Schmid KJ (2012) Islands and streams: clusters and gene flow in wild barley populations from the levant. Mol Ecol 21:1115–1129

Inostroza L, del Pozo A, Matus I, Castillo D, Hayes P, Machado S, Corey A (2009) Association mapping of plant height, yield, and yield stability in recombinant chromosome substitution lines (RCSLs) using *Hordeum vulgare* subsp. *spontaneum* as a source of donor alleles in a *Hordeum vulgare* subsp. *vulgare* background. Mol Breed 23:365–376

Ivandic V, Hackett CA, Nevo E, Keith R, Thomas WHB, Forster BP (2002) Analysis of simple sequence repeats (SSRs) in wild barley from the Fertile Crescent: Associations with ecology, geography and flowering time. Plant Mol Biol 48:511–527

Kasha KJ, Kao KN (1970) High frequency of haploid production in barley (*Hordeum vulgare* L.). Nature 225:874–876

Khlestkina EK, Varshney RK, Röder MS, Graner A, Börner A (2006) A comparative assessment of genetic diversity in cultivated barley collected in different decades of the last century in Austria, Albania and India by using genomic and genic SSR markers. Plant Genet Resour 4:125–133

Knüpffer H, van Hintum T (2003) Summarised diversity – the barley core collection. In: von Bothmer R, van Hintum T, Knüpffer H, Sato K (eds) Diversity in barley (*Hordeum vulgare*). Elsevier Science B.V., Amsterdam, pp 259–267

Koebner RMD, Dononi P, Reeves JC, Cooke RJ, Law JR (2003) Temporal flux in the molecular diversity of UK barley. Theor Appl Genet 106:550–558

Kolodinska AB, von Bothmer R, Dayteg C, Rashal I, Tuvesson S, Weibull J (2007) Genetic diversity changes and relationships in spring barley (*Hordeum vulgare* L.) germplasm of Nordic and Baltic areas as shown by SSR markers. Genet Resour Crop Evol 54:749–758

Kota R, Rudd S, Facius A, Kolesov G, Thiel T, Zhang H, Stein N, Mayer K, Graner A (2001a) Snipping polymorphisms from large EST collections in barley (*Hordeum vulgare* L.). Mol Genet Genom 270:24–33

Kota R, Varshney RK, Thiel T, Dehmer K, Graner A (2001b) Generation and comparison of EST-derived SSR and SNP markers in barley (*Hordeum vulgare* L.). Hereditas 135:141–151

Lee JM, Davenport GF, Marshall D, Ellis THN, Ambrose MJ, Dicks J, van Hintum TJL, Flavell AJ (2005) Germinate – a generic database for plant genetic resources. Plant Physiol 139:619–631

Malysheva-Otto LV, Ganal MW, Röder MS (2006) Analysis of molecular diversity, population structure and linkage disequilibrium in a worldwide survey of cultivated barley germplasm (*Hordeum vulgare* L.). BMC Genet 7:6

Malysheva-Otto L, Ganal MW, Law JR, Reeves JC, Röder MS (2007) Temporal trends of genetic diversity in European barley cultivars (*Hordeum vulgare* L.). Mol Breed 20:309–322

Matus IA, Hayes PM (2002) Genetic diversity in three groups of barley germplasm assessed by simple sequence repeats. Genome 45:1095–1106

Matus I, Corey A, Filichkin T, Hayes PM, Vales MI, Kling J, Riera O, Sato K, Powell W, Waugh R (2003) Development and characterization of recombinant chromosome substitution lines (RCSLs) using *Hordeum vulgare* subsp. *spontaneum* as a source of donor alleles in a *Hordeum vulgare* subsp. *vulgare* background. Genome 46:1010–1023

Moragues M, Comadran J, Waugh R, Milne I, Flavell AJ, Russell JR (2010) Effects of ascertainment bias and marker number on estimations of barley diversity from high throughput SNP genotype data. Theor Appl Genet 120:1525–1534

Naeem R, Dahleen L, Mirza B (2011) Genetic differentiation and geographical relationship of Asian barley landraces using SSRs. Genet. Mol Biol 34:268–273

Orabi J, Backes G, Wolday A, Yahyaoui A, Jahoor A (2007) The Horn of Africa as a centre of barley diversification and a potential domestication site. Theor Appl Genet 114:1117–1127

Pickering RA (1984) The influence of genotype and environment on chromosome elimination in crosses between *Hordeum vulgare* × *H. bulbosum*. Plant Sci 34:153–164

Pickering R (2000) Do the wild relatives of cultivated barley have a place in barley improvement? In: Logue S (ed) Barley genetics VIII: Proceedings of the 8th international barley genetics symposium, vol I, Department of Plant Science, Waite Campus, Adelaide University, Australia, pp 223–230

Pillen K, Binder A, Kreuzkam B, Ramsay L, Waugh R, Forster J, Leon J (2000) Mapping new EMBL-derived barley microsatellites and their use in differentiating German barley cultivars. Theor Appl Genet 101:652–660

Pillen K, Zacharias A, Leon J (2003) Advanced backcross QTL analysis in barley (*Hordeum vulgare* L.). Theor Appl Genet 107:340–352

Pillen K, Zacharias A, Leon J (2004) Comparative AB-QTL analysis in barley using a single exotic donor of *Hordeum vulgare* ssp. *spontaneum*. Theor Appl Genet 108:1591–1601

Rostoks N, Mudie S, Cardle L, Russell J, Ramsay L, Booth A, Svensson JT, Wanamaker S, Walia H, Rodriguez EM, Hedley PE, Liu H, Morris J, Close TJ, Marshall DF, Waugh R (2005) Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. Mol Gen Genomics 274:515–527

Russell J, Fuller J, Young G, Thomas B, Taramino G, Macaulay M, Waugh R, Powell W (1997) Discriminating between barley genotypes using microsatellite markers. Genome 40:442–450

Russell R, Ellis RP, Thomas WTB, Waugh R, Provan J, Booth A, Fuller JD, Lawrence PE, Young GR, Powell W (2000) A retrospective analysis of spring barley germplasm develoment from 'foundation genotypes' to currently successful cultivars. Mol Breed 6:553–568

Russell JR, Booth A, Fuller JD, Baum M, Ceccarelli S, Grando S, Powell S (2003) Patterns of polymorphism detected in the chloroplast and nuclear genomes of barley landraces sampled from Syria and Jordan. Theor Appl Genet 107:413–421

Russell J, Booth A, Fuller J, Harrower B, Hedley P, Machray G, Powell W (2004) A comparison of sequence-based polymorphism and haplotype content in transcribed and anonymous regions of the barley genome. Genome 47:389–398

Russell J, Dawson IK, Flavell AJ, Steffenson B, Weltzien E, Booth A, Ceccarelli S, Grando S, Waugh R (2011) Analysis of more than 1,000 SNPs in geographically-matched samples of landrace and wild barley indicates secondary contact and chromosome-level differences in diversity around domestication genes. New Phytologist 191:564–578

Saghai-Maroof MA, Biyashev RM, Yang GP, Zhang Q, Allard RW (1994) Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. Proc Natl Acad Sci 91:5466–5470

Sato K, Takeda K (2009) An application of high-throughput SNP genotyping for barley genome mapping and characterization of recombinant chromosome substitution lines. Theor Appl Genet 119:613–619

Sato K, Matsumoto T, Ooe N, Takeda K (2009) Genetic analysis of seed dormancy QTL in barley. Breed Sci 59:645–650

Sato K, Close TJ, Bhat P, Muñoz-Amatriaín M, Muehlbauer GJ (2011) Development of genetic map and alignment of recombinant chromosome substitution lines from a cross of EST donors by high accuracy SNP typing in barley. Plant Cell Physiol 52:728–737

Schmalenbach I, Korber N, Pillen K (2008) Selecting a set of wild barley introgression lines and verification of QTL effects for resistance to powdery mildew and leaf rust. Theor Appl Genet 117:1093–1106

Struss D, Plieske J (1998) The use of microsatellite markers for detection of genetic diversity in barley populations. Theor Appl Genet 97:308–315

Thiel T, Michalek W, Varshney RK, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor Appl Genet 106:411–422

van Hintum T, Menting F (2003) Diversity in *ex situ* genebank collections of barley. In: von Bothmer R, van Hintum T, Knüpffer H, Sato K (eds) Diversity in barley (*Hordeum vulgare*). Elsevier Science B.V., Amsterdam, pp 247–257

Varshney RK, Chabane K, Hendre PS, Aggarwal RK, Graner A (2007) Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys. Plant Sci 173:638–649

Varshney RK, Baum M, Guo P, Grando S, Ceccaeelli S, Graner A (2010) Features of SNP and SSR diversity in a set of ICARDA barley germplasm collection. Mol Breed 26:229–242

von Bothmer R, Jacobsen N (1985) Origin, taxonomy, and related species. In: Rasmusson D (ed) Barley. ASA Monograph No 26, pp 19–56

von Bothmer R, Linde-Laursen I (1989) Backcrosses to cultivated barley (*Hordeum vulgare* L.) and partial elimination of alien chromosomes. Hereditas 111:145–147

von Bothmer R, Flink J, Jacobsen N, Kotimäki M, Landström T (1983) Interspecific hybridization with cultivated barley (*Hordeum vulgare* L.). Hereditas 99:219–244

von Bothmer R, Jacobsen N, Baden C, Jørgensen RB, Linde-Laursen I (1995) An ecogeographical study of the genus *Hordeum*, 2nd edn. Systematic and ecogeographic studies on crop gene pools No 7. International Board of Plant Genetic Resources, Rome, 129 pp

von Bothmer R, Sato K, Komatsuda T, Yasuda S, Fischbeck G (2003) The domestication of cultivated barley. In: von Bothmer R, van Hintum T, Knüpffer H, Sato K (eds) Diversity in barley (*Hordeum vulgare*). Elsevier Science B.V., Amsterdam, pp 9–27

von Korff M, Wang H, Leon J, Pillen K (2004) Development of candidate introgression lines using an exotic barley accession (*Hordeum vulgare* ssp. *spontaneum*) as donor. Theor Appl Genet 109:1736–1745

Yahiaoui S, Igartua E, Moralejo M, Ramsay L, Molina-Cano JL, Ciudad FJ, Lasa JM, Gracia MP, Casas AM (2008) Patterns of genetic and eco-geographical diversity in Spanish barleys. Theor Appl Genet 116:271–282

Yun SJ, Gyenis L, Bossolini E, Hayes PM, Matus I, Smith KP, Steffenson BJ, Tuberosa R, Muehlbauer GJ (2006) Validation of quantitative trait loci for multiple disease resistance in barley using advanced backcross lines developed with a wild barley. Crop Sci 46:1179–1186

Chapter 3
Domestication

Takao Komatsuda

3.1 Introduction

Domestication refers to the deliberate cultivation of a wild plant (or taming of a wild animal) for human benefit. The selection of particular characteristics compatible with agricultural systems results in an evolutionary transition from a wild to a cultivated type. Only a small number of the over 300,000 extant plant species (Gornall 1997) have been domesticated. The reason why this number has remained so small reflects some intrinsic characteristics of the plants themselves, along with the particular environmental requirements for human existence (Gepts 2004). The plant type of the immediate wild ancestors of most of our current crop species was probably fairly close to the domesticated form. Domestication syndrome traits are shared between many of our crop species (Fuller 2007). Chief among these, in relation to species where the seed is the harvested product, are the absence of seed shattering, a greatly reduced degree of dormancy and a major increase with respect to both seed size and number (Zohary and Hopf 2000).

For long established species such as barley, domestication, as suggested by Harlan (1961, 1968), was a process rather than a single event, taking place in a diffused manner with respect to both space and time. The archaeological record has been quite informative in determining the time elapsed between the initial cultivation of a crop ancestor and the fixation of domestication-associated genes (Gepts 2004). Fuller and Allaby (2009) proposed a three stage model for the crop domestication process. In the first stage, selection is driven by polygenes controlling germination and seed size. In the second stage, natural selection acts on seed dispersal mechanisms (e.g. awnlessness in the cereals) and increases the size of the dispersal unit. Finally, during the third stage, harvesting results in the loss of

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wild-type dispersal mechanisms (e.g. shattering), which are typically under simple genetic control. The domestication process is most easily tracked in the cereals (Gepts 2004), where the domestication syndrome includes an expansion in caryopsis size, the evolution of a tough rachis (non-shattering) and the detachment of the caryopsis from the hull ('free-threshing').

3.2 The Triticeae Tribe

The Triticeae tribe includes a large number of temperate grasses and notably the economically important cereals wheat (*Triticum aestivum*), rye (*Secale cereale*) and barley (*Hordeum vulgare*). Current taxonomic understanding recognises some 30 genera within the tribe, all of which share the same basic chromosome number of $x = 7$. Ploidy levels range from diploid to at least decaploid of *Thinopyrum* sp. (10×), and at least 28 distinct genomes have been described (Barkworth and von Bothmer 2009). The inflorescence form comprises a spike (sometimes referred to as an 'ear'), in contrast to the panicle formed by most members of the related Aveneae, Bromeae and Poeae tribes (Clayton 1990). The spike of the majority of Triticeae species produces a single spikelet per rachis node, but in a few species, as many as three spikelets per node can develop. Each spikelet forms one to a few florets, each of which comprises a lemma, a palea, three anthers and a multibranched pistil. Two distinct forms of mature spike disarticulation have been defined. The first involves breakage of the rachis either immediately above or below the node (Sakuma et al. 2011); in either case, each disarticulated rachis fragment carries one (or a group of) spikelet(s). In some *Elymus*, *Aegilops* and *Triticum* species, breakage of the rachis at the basal node results in the intact spike falling to the ground at maturity. A correlation has been established between rachis fragility and the extent of the constriction around the rachis node, but the recognition of a true abscission layer has been difficult in the genera *Hordeum*, *Triticum* and *Aegilops* (Matsumoto et al. 1963; Ubisch 1915). The second form of spike disarticulation involves breakage of the rachilla, most commonly above the glume, although exceptionally it can also occur below the glume (as in *Elytrigia repens*) or even between florets (e.g. *Hystrix* spp.). The genera *Elymus*, *Elytrigia*, *Aegilops*, *Triticum*, *Hordeum* and *Eremopyrum* include species of both disarticulation types (Sakuma et al. 2011).

3.3 The Genus *Hordeum*

The genus *Hordeum* consists of 31 or 33 species endemic to the Northern Hemisphere, southern Africa and the southern cone of South America (Blattner 2009; Bothmer et al. 1995). *H. murinum* and *H. marinum* are globally distributed weeds found in many agricultural habitats. The range in ploidy level is from diploid to hexaploid and involves combinations of the four basic genomes designated by

Wang et al. (1994) as I (*H. vulgare* and *H. bulbosum*), Xu (*H. murinum*), Xa (*H. marinum*) and H (all the remaining species). Phylogenetic analysis based on DNA sequence has shown that the I genome is related to Xu and H to Xa. Both the I/Xu and the Xa/H groups are thought to be monophyletic. *H. marinum* includes the two subspecies *gussoneanum* and *marinum*. The former is the donor of the Xa genome and is represented in the allopolyploids *H. marinum* subsp. *gussoneanum* (4×), *H. secalinum* (4×), *H. capense* (4×) and *H. brachyantherum* (6×) (Komatsuda et al. 2001, 2009; Petersen and Seberg 2004; Sun et al. 2009), all of which also carry the *gussoneanum* cytoplasm (Jakob and Blattner 2006; Nishikawa et al. 2002). The outcome of these studies suggests that *H. marinum* subsp. *gussoneanum* possibly carries a gene which promotes the formation of interspecific hybrids and subsequent polyploidization, leading to the formation of the polyploidy taxa. Uncommonly for Triticeae species, all *Hordeum* species develop three spikelets per rachis node, comprising one central and two laterals. Each spikelet forms only a single floret. Disarticulation in all species except for *H. bogdanii* occurs above the rachis node to produce wedge-type spikelets (Bothmer 1979; Sakuma et al. 2011). Seed dispersal has evolved in two directions. One favours wind dispersal (*H. jubatum*, *H. lechleri* and *H. comosum*), where very small, light caryopses are attached to a long slender awn, while other (*H. vulgare*, *H. bulbosum* and *H. murinum*) relies on animal carriers, forming large, heavy caryopses (Bothmer et al. 1995).

3.4 Wild Barley

Given that wild barley (formerly classified as *H. spontaneum*) and cultivated barley are fully interfertile, the two forms are now treated as subspecies (subsp. *spontaneum* (C. Koch) Thell. and subsp. *vulgare*, respectively) (Bothmer et al. 1995). They are similar to one another at the morphological level, but the rachis of subsp. *spontaneum* is brittle and the spike is always two rowed. The natural distribution of subsp. *spontaneum* covers parts of Greece, Turkey, Iran, Iraq, Afghanistan, Syria, Jordan and Israel (Bothmer et al. 2003). It was first discovered in Turkey by the German botanist Carl Koch and was immediately recognised to be the immediate ancestor of cultivated barley. The subspecies is adapted to a broad range of environments and is particularly regarded as a fruitful source of genetic variation with respect to drought and salinity tolerance (Nevo and Chen 2010). Its drought adaptive traits include reduced height, earliness, peduncles and peduncle extrusion (Shakhatreh et al. 2010).

3.5 Archaeological Evidence for the Cultivation of Barley and Its Geographical Spread

The disarticulation scars of subsp. *spontaneum* are smooth, whereas the mechanical threshing of subsp. *vulgare* produces a rough scar on the surface of the grain. This feature therefore serves as a diagnostic of the presence of subsp. *vulgare* in archaeological grain specimens (Zohary and Hopf 2000). The oldest known specimens were recovered from Ohalo II, a preagricultural site on the south shore of the Sea of Galilee, and have been dated to 17000 Before Common Era (BCE) (Kislev et al. 1992), cited by Zohary and Hopf (2000). The grains were morphologically identical to those of modern subsp. s*pontaneum*. The earliest proven remains of subsp. *vulgare* have been dated to the period 7500–6400 BCE. These are typically found in admixtures with subsp. *spontaneum* grain (Zohary and Hopf 2000). Field experiments based on harvesting einkorn wheat (*T. monococcum*) suggest that the most efficient ancient system for harvesting cereals was likely to have involved sickle reaping of plants with a tough rachis (Hillman and Davies 1990). Other possible systems include beating and uprooting.

The absence of the wild-type seed dispersal mechanism is one of the most important domestication syndrome traits. In the cereals, this is achieved by the loss of the abscission layer close to the rachis node. It was long held that selection pressure for this loss was imposed by the use of the sickle for harvesting, but Fuller and Allaby (2009) have suggested that this is unlikely, given that the non-shattering habit appears to have evolved more slowly than might be predicted by the sickle harvesting model. The domestication of wheat was slow following the outset of its pre-domestication cultivation (Tanno and Willcox 2006), and the process was similarly slow in barley (Fuller 2007; Tanno and Willcox 2012). All subsp. *spontaneum* accessions form a two-rowed spike, which is therefore taken to be the ancestral form. The alternative six-rowed spike is clearly less well adapted for survival in the wild, because spontaneous mutants appear to be rapidly eliminated from wild populations (Zohary 1964). Only the central spikelet is fertile, but together the three spikelets form a light, arrowhead-like dispersal unit which both facilitates its dispersal by animals and its eventual burial. The many upward pointing barbs on the lemma and awn also form a part of the dispersal and self-burial apparatus. In wild wheats, the awns too help the spikelet work its way into the soil (Elbaum et al. 2007).

The cultivation of six-rowed barley started around 6800–6000 BCE, and by 5000–4000 BCE, it featured in the alluvial soils of Mesopotamia and Lower Egypt. Six-rowed barley soon replaced two-rowed types and established itself as the most important crop of the Neolithic civilizations of the Middle East (Zohary and Hopf 2000). The selection and propagation of six-rowed types was an important part of the domestication process (Harlan and Wet 1973). Their spikes potentially set three times as many grains as the two-rowed spike, although six-rowed plants tend to tiller less freely and their grains are smaller than those set by two-rowed plants. The yield advantage of the six-rowed type, in terms of weight of grain per

unit area, is not marked. However, the higher number of grain harvested per unit area is advantageous in terms of grain production for subsequent sowing. Archaeological specimens of six-rowed barley have been found in the aceramic Neolithic beds in Tell Abu Hureyra, dating to 6800 BCE (Helbaek 1959). Two-rowed barley grains from the same site are slightly older (about 7000 BCE) and are typically found alongside a minor component of six-rowed barley grain (Helbaek 1969). The early farmers spread from the Fertile Crescent in a NW direction, so that agriculture penetrated the European continent over the period 7000–3000 BCE. Both two- and six-rowed barleys emerged in Greece between 6000 and 4000 BCE, but over most of the Mediterranean Basin, six-rowed barleys predominated (Zohary and Hopf 2000). Once the two-rowed types had disappeared from ancient Mesopotamia and Egypt, they did not re-emerge in the archaeological record in these regions until around 1000 CE (Helbaek 1959). Their cultivation in central and northern Europe was unknown until around 1,000 years ago, possibly having been introduced by returning Crusaders (Fischbeck 2002). The mutations required for the loss of the winter habit (vernalization non-responsiveness) are thought to have occurred post-domestication, while critical mutations affecting photoperiod sensitivity occurred pre-domestication (Cockram et al. 2011).

3.6 Genetic Inferences Relating to Barley Domestication

Takahashi (1955) took the presence of the two non-brittle rachis genes (*btr1* and *btr2*) to imply that two independent domestication processes occurred in barley. The two domestication hypothesis was supported by the geographical separation of barley germ plasm between the Middle East/Europe/North Africa and eastern Asia. The two gene pools can be readily differentiated from one another by their DNA sequence at five nuclear loci and the expression of two morphological traits (Saisho and Purugganan 2007). The conclusion drawn from the sequence data was that while European and North African barleys originated largely from the Fertile Crescent, the South and East Asia landraces more likely arose from populations endemic to the eastern edge of the Iranian Plateau. DNA sequence analysis of the genomic region surrounding *Btr1* and *Btr2* has confirmed that independent mutations were responsible for the trait in the two gene pools (Azhaguvel and Komatsuda 2007), and a phylogenetic analysis based on multilocus markers mapping to the region of the *Btr* genes was consistent with the two independent domestication processes theory (Komatsuda et al. 2004). When the two domestication hypothesis was tested on a larger set of genes, Morrell and Clegg (2007) concluded that the western gene pool arose from the Fertile Crescent and the eastern one from populations sited some 1,500–3,000 km to the East. In contrast, the alternative hypothesis holds that there was just a single barley domestication process, which occurred in the modern-day border region between Israel and Jordan (Badr et al. 2000; Salamini et al. 2002). The basis for this idea is the analysis of genome-wide DNA polymorphisms. However, as Allaby et al. (2008) have shown

that genome-wide marker systems are likely to predict monophyletic clades in crops with known multiple origins, the support for the one domestication hypothesis has become rather weak. AFLP genotyping has been used to generate two rather distinct phylogenies, one based on a whole genome scan and the other on markers mapping to the regions associated with the non-brittle rachis genes. The latter phylogeny implied the occurrence of two separate barley domestication events, as it showed a clear separation between the western and eastern gene pools (Komatsuda et al. 2004). This same separation was supported by further data provided by Azhaguvel and Komatsuda (2007). Some doubt has been cast as to whether subsp. *spontaneum* as it exists today in the Fertile Crescent is the progenitor of subsp. *vulgare* types which emerged in the Horn of Africa (Orabi et al. 2007).

3.7 Domestication Genes

3.7.1 Non-brittle Rachis

Over 90 % of the rachis nodes on the subsp. *spontaneum* spike are brittle at maturity. Disarticulation starts from the tip of the spike and moves downward to its base, where grains remain attached to the lowest rachis node. The presence of a dominant allele at each of the two genes *Btr1* and *Btr2* is required for the expression of the brittle rachis trait (Takahashi and Hayashi 1964), with the loss-of-function mutation at either (or both) resulting in the formation of a non-brittle rachis. The genotype *btr1Btr2* dominates in the western subsp. *vulgare* gene pool, while most East Asian cultivars are *Btr1btr2* (Takahashi 1955). The two loci are tightly linked with one another on the short arm of chromosome 3H (Komatsuda and Mano 2002; Takahashi and Hayashi 1964). As yet neither gene has been isolated, although the genetic window defining the location of *Btr1* has been narrowed to just 0.8 cM (Azhaguvel et al. 2006). In addition to the *Btr1*/*Btr2* complementary gene pair, a quantitative trait locus (QTL) affecting disarticulation has been detected on each of chromosomes 5H and 7H (Komatsuda and Mano 2002; Komatsuda et al. 2004). The latter, temporarily denoted as '*D*', has the stronger effect. Its wild-type (subsp. *spontaneum*) allele may be necessary for the proper formation of the abscission layer. *D* maps in the vicinity of *dense spike 1* (*dsp1*), which may suggest its effect is a pleiotropic consequence of *dsp1* action, rather than reflecting the presence of an independent gene. The presence of the recessive allele at *dsp1* is associated with a reduced percentage of rachis which is brittle; however, while the semi-brachytic recessive mutation *uzu* also produces a dense spike, it has no equivalent effect on the percentage of rachis which is brittle (Komatsuda et al. 2004; Senthil and Komatsuda 2005). As the chromosome 5H QTL maps to the long arm of the chromosome (Komatsuda et al. 2004), it may be an orthologue of the wheat gene *Q*, which determines the free-threshing habit and maps to chromosome 5AL. In the presence of *q*, the wheat spike is speltoid and disarticulates into wedge-type

spikelets. The isolation of *Q* showed that it encodes a member of the AP2-like family of transcription factors (Faris et al. 2003; Simons et al. 2006).

In a number of the Triticeae species, the brittle rachis trait is determined by a gene(s) located on their homoeologous group 3 chromosomes [reviewed by Sakuma et al. (2011)]. The relevant chromosome(s) in *T. timopheevii* (Li and Gill 2006), *T. turgidum* ssp. *dicoccoides* (Nalam et al. 2006) and *T. aestivum* (Chen et al. 1998) all carry genes responsible for disarticulation above the rachis node, as in barley. In *Ae. tauschii*, the progenitor of the bread wheat D genome, disarticulation occurs below the rachis node and is controlled by a gene(s) carried on the long (rather than the short) arm of chromosome 3D (Li and Gill 2006). This gene acts hypostatically to the gene controlling whole spike disarticulation at the basal node of the spike, but although a large number of synthetic hexaploid (*T. turgidum* ssp. *durum* × *Ae. tauschii*) wheats have been produced, none have been reported to express the brittle rachis trait. The 3DL gene may nevertheless belong to the same orthologous set, given that intra-chromosomal translocations are not uncommon during evolution (Devos et al. 1993; Liu et al. 1992; Zhang et al. 2001). The rice QTL *qSH1*, which has been shown to encode a BEL1-type homoeobox protein, produces an abscission layer above the glume (Konishi et al. 2006). *qSH1* lies on a region of chromosome 1 which shares synteny with barley chromosome 3H (Stein et al. 2007), and a presumed barley orthologue *HvJuBel2* has been identified on 3HL (Muller et al. 2001). However, since the *Btr* genes map to 3HS (Komatsuda and Mano 2002), this excludes the possibility that *JuBel2* as a *Btr* candidate. (Nevertheless, it remains possible that the *AtJuBel2* orthologue, if one is present, may represent the brittle rachis gene mapping on *Ae. tauschii* 3DL.) Some further sources of relevant variation in barley are represented by the 'head shattering' QTL mapped to chromosome 3H by Kandemir et al. (2000) and by a chromosome 1H gene in subsp. *spontaneum* where the presence of a recessive allele confers a difficult-to-thresh spike in which the rachis and awns were only partially removed after mechanical threshing (Schmalenbach et al. 2011). The extensive collection of barley mutants also includes three 'brittle culm' types, in which certain cellulose-synthesising enzyme complexes are deficient (Kimura et al. 1999).

3.7.2 Six-Rowed Spike

The ancestral two-rowed spike in subsp. *spontaneum* and two-rowed subsp. *vulgare* cultivars is controlled by a dominant allele at the *vrs1* locus on chromosome arm 2HL. Komatsuda et al. (2007) have shown that the six-rowed type is determined by at least three independent point mutations in *Vrs1*. The first of these (*vrs1.a1*) is a single nucleotide deletion which induces a frame shift, the second (*vrs1.a2*) an insertion event causing a frame shift mutation and the third (*vrs1.a3*) a nucleotide substitution which generates a non-synonymous amino acid substitution in the VRS1 homeodomain. The *Vrs1.b3* allele is widely distributed among two-rowed

cultivars and appears to have arisen rather recently from wild barley as demonstrated by the identification of just one haplotype among cultivars and wild barley (Saisho et al. 2009). It has been suggested that the use of two-rowed barley spreads rapidly throughout the world, coming to dominate the crop's production during the medieval period (Fischbeck 2002). The identification of just one haplotype among *vrs1.a3* type six-rowed cultivars suggested that its origin must have been rather recent (Saisho et al. 2009). Many de novo mutants producing a six-rowed spike can be ascribed to sequence alteration(s) within *Vrs1* (Komatsuda et al. 2007). The dominant allele at *Vrs1* suppresses the development of the lateral spikelets. The isolation of this gene has shown that the subsp. *spontaneum Vrs1* allele encodes a transcription factor including a homeodomain (HD) and leucine zipper (Zip) (Komatsuda et al. 2007). *Vrs1* has no clear homologue in *Brachypodium distachyon*, rice or maize, but *HvHox2*, a *Vrs1* paralogue on chromosome arm 2HS, is well conserved among the cereals as well as in other plant species (Pourkheirandish et al. 2007; Sakuma et al. 2010). The indication is that *Vrs1* arose from a duplication of *HvHox2*, which was subsequently translocated from the short arm to the long arm of chromosome 2H. *Vrs1* expression is restricted to the lateral spikelets, while that of *HvHox2* is ubiquitous. Based on the hypothesis that HvHOX2 and VRS1 share the same target DNA sequence and retain the same level of affinity, it has been proposed by Sakuma et al. (2010) that VRS1 competes with HvHOX2 to bind to a *cis*-element(s) within a downstream gene(s). The formation of HvHOX2/VRS1 heterodimers would then drive down the population of HvHOX2 homodimers, so that the stronger the expression of *Vrs1*, the more the action of *HvHox2* will be suppressed. VRS1 may act as a repressor of the downstream gene (s), because VRS1 has lost the C-terminal motif postulated to act as a transcription activator.

The resequencing of the *Vrs1* sequence in a large collection of barley cultivars resulted in the identification of a further haplotype among six-rowed types (Saisho et al. 2009). Since its deduced peptide sequence was identical to that generated from *Vrs1.b2*, the awnless lateral spikelet produced by *vrs1.c* cannot be explained by a mutation in the *Vrs1* coding sequence. Instead, it was proposed that *Vrs1* transcript abundance was either reduced or the transcript itself was made less stable as a result of mutations in either the promoter or the downstream regions of the gene. Partial resequencing of the critical region of the *vrs1.c* gene generated the novel haplotype *vrs1.a4* (Cuesta-Marcos et al. 2010), although the variant bases were not considered to be responsible for the altered phenotype. *Lks1* is an awnless mutant affecting both the central and lateral spikelets. The mutation is closely—but not completely—linked to *Vrs1* (Lundqvist et al. 1997), and the mutant line carries the *vrs1.a4* haplotype (Saisho et al. 2009), which was taken to suggest that *Lks1* is either a *cis*-element of *Vrs1* or that it encodes a transacting factor of *Vrs1* or other six-rowed spike genes. Unlike the two-rowed type, the *deficiens* type (*Vrs1.t*) does develop at least rudimentary lateral spikelets. Its VRS1 product includes a serine to glycine shift at position 184 of motif 3, but is otherwise identical to that of the *Vrs1.b2* product (Saisho et al. 2009). It has yet to be established whether or not this polymorphism is responsible for the phenotype. In the *irregulare* type, each rachis

node produces between one and three spikelets, where always the central is produced, but otherwise rule was not clear indicating *irregulare* stochastically determines the development of lateral spikelets. All *irregulare* accessions share the *vrs1.a1* haplotype (Saisho et al. 2009; Youssef et al. 2012).

A mutation in *Vrs1* in itself is insufficient to generate a six-rowed spike. Allelic variation at the *I* genes on the short arm of chromosome 4H is involved in determining the extent to which grains develop in the lateral spikelet. Most six-rowed cultivars carry either *I* or I^h (Leonard 1942; Woodward 1947). The *intermedium spike c* (*int-c*) mutation (Fukuyama et al. 1982; Lundqvist 1991; Lundqvist and Lundqvist 1988), as represented by over 20 induced mutant lines (Lundqvist and Lundqvist 1988), involves a gene located on the short arm of chromosome 4H (Lundqvist et al. 1997). In the presence of the recessive *int-c* allele, the lateral spikelets become at least partially fertile, thereby producing the *intermedium* type spike. In contrast, it is the dominant alleles at *I* which promote the development of the lateral spikelets. It has been suggested that *I* and *int-c* are allelic with one another (Lundqvist et al. 1997), but the opposite direction of their dominance would argue against this. The *int-c* gene is a homologue of the maize gene *TEOSINTE BRANCHED 1*, a member of the *TCP* gene family, which contains genes putatively encoding basic helix-loop-helix DNA-binding proteins and which is involved in the control of organ growth (Ramsay et al. 2011). The phenotype associated with the *int-c* mutation can be explained by a loss-of-function in a *TCP* gene, but the same cannot be applied to the *I* gene. It remains to be determined which *I* allele is carried by subsp. *spontaneum*. A resequencing exercise of the *int-c locus* based on a set of >200 accessions of *irregulare* or *labile* barleys indigenous to Ethiopia showed that half of *labile* barleys appeared to carry a duplication (Youssef et al. 2012).

3.7.3 Reduced Dormancy

Dormancy—the inability to germinate under favourable germination conditions—is particularly marked in subsp. s*pontaneum*, a feature which has presumably evolved to avoid early germination during the hot, dry period between maturity and the onset of the more reliable winter rainfall. Extreme dormancy is a problem for a crop situation, both with respect to crop establishment and, in the case of barley in particular, for the malting process. Fully domesticated plants display little or no dormancy compared to their wild progenitors. The dormant barley caryopsis can survive periods of prolonged drought and/or high temperature, whereas the seedling is highly sensitive to both of these stresses. In subsp. s*pontaneum*, it has been possible to establish a negative correlation between the strength of dormancy and the ability of seedlings to recover from an episode of drought, and it was suggested therefore that the trade-off between these two traits may reflect differences in hormone levels in the caryopsis and the seedling (Zhang and Gutterman 2003). Stringent selection against dormancy, however, produces increased

susceptibility to preharvest sprouting (Prada et al. 2004), which is a highly undesirable trait. Ethiopian cultivars in general appear to show very low levels of dormancy.

The genetic control of dormancy has largely been determined via QTL analysis. The locus *SD1*, mapping to the centromeric region of chromosome 5H (Han et al. 1999; Prada et al. 2004; Zhang et al. 2005), has a large effect (explaining 30–50 % of the phenotypic variance in a number of mapping populations) (Hori et al. 2007). A fine map of *SD1* was constructed (Sato et al. 2009), but the gene has not yet been isolated. *SD2* is responsible for a somewhat lesser level of dormancy, which is attractive in the context of barley improvement (Gao et al. 2003). This locus has been mapped within a distally located 0.8 cM segment of chromosome arm 5HL (Gao et al. 2003; Hori et al. 2007). Li et al. (2004) proposed that a gene within this segment encoding gibberellic acid (GA) 20 oxidase was a likely candidate for *SD2*, but this suggestion has yet to be verified. A QTL involved in the control of preharvest sprouting and seed dormancy—presumably identical to *SD2*—was mapped by Zhang et al. (2011) to the distal tip of chromosome 5HL. *SD1* may act epistatically to *SD2* at the early ripening stage, but the interaction appears to be additive at the later stages (Romagosa et al. 1999). Specific mapping populations have been generated to identify QTL affecting preharvest sprouting on chromosomes 1H, 2H, 3H and 7H and those affecting seed dormancy on chromosomes 1H, 2H and 7H (Ullrich et al. 2009).

Phytohormones play a key role in maintaining and breaking dormancy. High levels of abscisic acid (ABA) and low ones of GA are associated with dormancy. The 9-cis-epoxycarotenoid dioxygenase (NCED) gene family plays a critical role with respect to the control of ABA levels in barley (Chono et al. 2006; Leymarie et al. 2008). White light promotes dormancy in freshly harvested cereal caryopses, and also enhances the expression of *HvNCED1* in the barley embryo, so that dormant grains imbibed under white light tend to accumulate more ABA in the embryo than those imbibed in the dark (Gubler et al. 2008). Dormancy seems to have little effect on the expression of ABA biosynthesis genes, but rather promotes the expression of an ABA catabolism gene and genes encoding the synthesis and catabolism of GA. RNA interference experiments in barley have demonstrated a role in dormancy release for the ABA catabolism gene *HvABA8'OH1* (Millar et al. 2006). The hydrogen peroxide molecule may also be implicated in the alleviation of dormancy via the activation of GA signalling and synthesis (Bahin et al. 2011). Nitric oxide is a further agent of dormancy reduction, both in the *Arabidopsis thaliana* seed and the barley caryopsis. Its effect on germination acts upstream of ABA action (Bethke et al. 2004). Comparative gene expression experiments involving dormant and nondormant embryos have identified a gene sharing homology to a fructose-6-phosphate-2-kinase/fructose-2,6-biphosphatase as a candidate for maintaining dormancy both in barley and in other cereals (Leymarie et al. 2007). Both wheat and barley homologues of the *A. thaliana* dormancy gene *DOG1* increase the level of dormancy when heterologously expressed in *A. thaliana* (Ashikawa et al. 2010), suggesting that they may have a dormancy-related function in the cereals as well. Oxygen interacts with ABA and GA to

regulate dormancy. The dormancy of the barley caryopsis is heavily influenced by the action of the hull to prevent the diffusion of oxygen into the embryo (Bradford et al. 2008).

3.7.4 Naked Caryopsis

Free-threshing barleys (where the hull does not adhere at maturity) are referred to as 'naked'. Hull adherence reflects the formation of a lipid layer between the pericarp epidermis and the hull. The naked caryopsis phenotype is determined by the recessive gene *nud*, caused by a 17 kbp deletion which includes a gene encoding an ethylene response factor family transcription factor (Taketa et al. 2008). The occurrence of naked caryopsis types dating to around 6000 BCE indicates that the deletion event was selected very early during the domestication of barley, although probably following the emergence of the six-rowed spike (Helbaek 1969). Naked barleys are cultivated in many parts of the world, but there is a particular preference for East Asia, especially in Tibet and northern Nepal, India and Pakistan (Bothmer et al. 2003).

3.7.5 Vernalization Requirement

Plants which need a period of low temperature to induce the switch from vegetative to reproductive growth (a 'vernalization requirement') are referred to as winter types. This property is achieved in subsp. *spontaneum* by the simultaneous presence of the dominant allele *Sgh1* (syn. *HvVrn2*), the recessive *sgh2* (syn. *Hvvrn1*) and the recessive *sgh3* (syn. *Hvvrn3*). A mutation to any one of these alleles proved sufficient to abolish the vernalization requirement and so to allow for spring sowing (Yasuda 1969). Spring barleys are common in agriculture: firstly, in areas where the winter conditions are too harsh to allow survival of autumn-sown plants (e.g. Canada) and, secondly, where the summer is too hot and dry (but the winter is mild) to permit adequate grain filling (e.g. Australia) (Bothmer et al. 2003). Vernalization treatment increases the level of histone 3 lysine 4 trimethylation and decreases that of histone 3 lysine 27 trimethylation in *HvVRN1* (Oliver et al. 2009). Its wheat orthologue encodes an AP1 MADS box product (Murai et al. 2003; Yan et al. 2003). Major deletions in the first intron of *HvVRN1* are associated with reversion to spring type in barley, wheat and perennial ryegrass (Asp et al. 2011; Fu et al. 2005; Zitzewitz et al. 2005). The presence of several different deletions shows that the mutation from winter to spring habit has occurred a number of times in an independent manner (Cockram et al. 2007). *VRN2* is a transacting repressor of *VRN1* and encodes a zinc finger and CCT domain protein (Yan et al. 2004); in wheat, *VRN1* and *VRN2* show contrasting transcription profiles, with *VRN2* being downregulated by cold treatment (Yan et al. 2004, 2006). *HvVRN2* is deleted in

spring-type barleys (Dubcovsky et al. 2005; Yan et al. 2004) and the deletion produced the facultative phenotype (Zitzewitz et al. 2005). The presence of the same deletion in all spring barleys suggests a single origin for this allele (Dubcovsky et al. 2005). The *VRN3* genes are homologues of *FT* (Yan et al. 2006), which in rice is said to determine the synthesis of the hormone 'florigen' (Taoka et al. 2011). The dominant allele produces a high level of transcription and accelerates flowering in both barley and wheat.

3.7.6 Photoperiod Insensitivity

Flowering time can also be affected by the photoperiod (Laurie 1997). Subsp. *spontaneum* generally requires a photoperiod of at least 12 h to trigger the switch to reproductive growth. The trait is governed by the presence of a dominant allele at the *Ppd-H1* gene on chromosome 2H, which encodes a pseudo-response regulator (Turner et al. 2005). The basis of photoperiod insensitivity, which has allowed the expansion of barley cultivation into higher latitudes, is a single nucleotide polymorphism (SNP79) (Turner et al. 2005), although Jones et al. (2008) have suggested that SNP48 is more strongly associated with the phenotype than SNP79. The origin of the photoperiod insensitive allele in European material is thought to be an Iranian population of subsp. *spontaneum* (Jones et al. 2008). Under long day conditions, *Ppd-H1* exerts a significant level of pleiotropy on plant height, spike length and grain yield and also influences tiller number and the number of grains produced per spike (Laurie et al. 1994; Sameri et al. 2006). A second important gene is *Ppd-H2*, which affects flowering time when plants are exposed to a day length under 10 h, but has little effect under long days (13–16 h) (Laurie et al. 1995; Szucs et al. 2006). *Ppd-H2* probably encodes the *FT*-like gene *HvFT3* (Faure et al. 2007; Kikuchi et al. 2009). Photoperiod interacts with vernalization to determine flowering time (Karsai et al. 2008; Trevaskis et al. 2006). This interaction operates via the integration of the low temperature and photoperiod response pathways to induce the transcription of *VRN3* via the induction of *VRN2* (Hemming et al. 2008).

References

Allaby RG, Fuller DQ, Brown TA (2008) The genetic expectations of a protracted model for the origins of domesticated crops. Proc Natl Acad Sci U S A 105:13982–13986

Ashikawa I, Abe F, Nakamura S (2010) Ectopic expression of wheat and barley DOG1-like genes promotes seed dormancy in Arabidopsis. Plant Sci 179:536–542

Asp T, Byrne S, Gundlach H, Bruggmann R, Mayer KF, Andersen JR, Xu M, Greve M, Lenk I, Lubberstedt T (2011) Comparative sequence analysis of VRN1 alleles of Lolium perenne with the co-linear regions in barley, wheat, and rice. Mol Genet Genomics 286:433–447

Azhaguvel P, Komatsuda T (2007) A phylogenetic analysis based on nucleotide sequence of a marker linked to the brittle rachis locus indicates a diphyletic origin of barley. Ann Bot (Lond) 100:1009–1015

Azhaguvel P, Vidya-Saraswathi D, Komatsuda T (2006) High-resolution linkage mapping for the non-brittle rachis locus btr1 in cultivated × wild barley (*Hordeum vulgare*). Plant Sci 170:1087–1094

Badr A, Müller K, Schäfer-Pregl R, El Rabey H, Effgen S, Ibrahim H, Pozzi C, Rohde W, Salamini F (2000) On the origin and domestication history of barley (*Hordeum vulgare*). Mol Biol Evol 17:499–510

Bahin E, Bailly C, Sotta B, Kranner I, Corbineau F, Leymarie J (2011) Crosstalk between reactive oxygen species and hormonal signalling pathways regulates grain dormancy in barley. Plant Cell Environ 34:980–993

Barkworth M, von Bothmer R (2009) Scientific names in the Triticeae. In: Feuillet C, Muehlbauer G (eds) Genetics and genomics of the *Triticeae*. Springer, New York, NY, pp 3–30

Bethke PC, Gubler F, Jacobsen JV, Jones RL (2004) Dormancy of Arabidopsis seeds and barley grains can be broken by nitric oxide. Planta 219:847–855

Blattner FR (2009) Progress in phylogenetic analysis and a new infrageneric classification of the barley genus Hordeum (Poaceae: Triticeae). Breed Sci 59:471–480

Bothmer R (1979) Revision of the Asiatic taxa of Hordeum sect. Stenostachys. Bot Tidsskr 74:117–147

Bothmer Rv, Jacobsen N, Baden C, Jørgensen RB, Linde-Laursen I (1995) An ecogeographical study of the genus Hordeum, 2nd edn. Systematic and ecogeographic studies on crop genepools 7. International Plant Genetic Resources Institute, Rome

Bothmer R, Sato K, Komatsuda T, Yasuda S, Fischbeck G (2003) The domestication of cultivated barley. In: von Bothmer R, van Hintum T, Knüpffer H, Sato K (eds) Diversity in barley (*Hordeum vulgare*). Elsevier, Amsterdam, pp 9–27

Bradford KJ, Benech-Arnold RL, Come D, Corbineau F (2008) Quantifying the sensitivity of barley seed germination to oxygen, abscisic acid, and gibberellin using a population-based threshold model. J Exp Bot 59:335–347

Chen Q-F, Yen C, Yang J-L (1998) Chromosome location of the gene for brittle rachis in the Tibetan weedrace of common wheat. Genet Resour Crop Evol 45:407–410

Chono M, Honda I, Shinoda S, Kushiro T, Kamiya Y, Nambara E, Kawakami N, Kaneko S, Watanabe Y (2006) Field studies on the regulation of abscisic acid content and germinability during grain development of barley: molecular and chemical analysis of pre-harvest sprouting. J Exp Bot 57:2421–2434

Clayton W (1990) The spikelet. In: Chapman G (ed) Reproductive versatility in the grasses. Cambridge University Press, Cambridge, pp 32–51

Cockram J, Chiapparino E, Taylor SA, Stamati K, Donini P, Laurie DA, O'Sullivan DM (2007) Haplotype analysis of vernalization loci in European barley germplasm reveals novel VRN-H1 alleles and a predominant winter VRN-H1/VRN-H2 multi-locus haplotype. Theor Appl Genet 115:993–1001

Cockram J, Hones H, O'Sullivan DM (2011) Genetic variation at flowering time loci in wild and cultivated barley. Plant Genet Resour Characterization Utilization 9:264–267

Cuesta-Marcos A, Szucs P, Close TJ, Filichkin T, Muehlbauer GJ, Smith KP, Hayes PM (2010) Genome-wide SNPs and re-sequencing of growth habit and inflorescence genes in barley: implications for association mapping in germplasm arrays varying in size and structure. BMC Genomics 11:707

Devos KM, Atkinson MD, Chinoy CN, Francis HA, Harcourt RL, Koebner RMD, Liu CJ, Masojc P, Xie DX, Gale MD (1993) Chromosomal rearrangements in the rye genome relative to that of wheat. Theor Appl Genet 85:673–680

Dubcovsky J, Chen C, Yan L (2005) Molecular characterization of the allelic variation at the VRN-H2 vernalization locus in barley. Mol Breed 15:395–407

Elbaum R, Zaltzman L, Burgert I, Fratzl P (2007) The role of wheat awns in the seed dispersal unit. Science 316:884–886

Faris J, Fellers J, Brooks S, Gill B (2003) A bacterial artificial chromosome contig spanning the major domestication locus Q in wheat and identification of a candidate gene. Genetics 164:311–321

Faure S, Higgins J, Turner A, Laurie DA (2007) The FLOWERING LOCUS T-like gene family in barley (Hordeum vulgare). Genetics 176:599–609

Fischbeck G (2002) Contribution of barley to agriculture: a brief overview. In: Slafer GA, Molina-Cano JL, Savin R, Araus JL, Romagosa I (eds) Barley science: recent advance from molecular biology to agronomy of yield and quality. Food Products Press, New York, NY, pp 1–14

Fu D, Szucs P, Yan L, Helguera M, Skinner JS, von Zitzewitz J, Hayes PM, Dubcovsky J (2005) Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. Mol Genet Genomics 273:54–65

Fukuyama T, Takahashi R, Hayashi J (1982) Genetic studies on the induced six-rowed mutants in barley. Ber Ohara Inst landwirtsch Biol Okayama Univ 18:99–113

Fuller DQ (2007) Contrasting patterns in crop domestication and domestication rates: recent archaeobotanical insights from the old world. Ann Bot 100:903–924

Fuller D, Allaby R (2009) Seed dispersal and crop domestication: shattering, germination and seasonality in evolution under cultivation. In: Østergaard L (ed) Fruit development and seed dispersal, vol 38, Annu Plant Rev., pp 238–295

Gao W, Clancy JA, Han F, Prada D, Kleinhofs A, Ullrich SE (2003) Molecular dissection of a dormancy QTL region near the chromosome 7 (5H) L telomere in barley. Theor Appl Genet 107:552–559

Gepts P (2004) Crop domestication as a long-term selection experiment. In: Janick J (ed) Plant breeding reviews, Part 2, vol 24. Wiley, Hoboken, NJ

Gornall R (1997) Practical aspects of the species concept in plants. In: Claridge M, Dawah H, Wilson M (eds) Species. Chapman & Hall, London, pp 171–190

Gubler F, Hughes T, Waterhouse P, Jacobsen J (2008) Regulation of dormancy in barley by blue light and after-ripening: effects on abscisic acid and gibberellin metabolism. Plant Physiol 147:886–896

Han F, Ullrich SE, Clancy JA, Romagosa I (1999) Inheritance and fine mapping of a major barley seed dormancy QTL. Plant Sci 143:113–118

Harlan J (1961) Geographic origin of plants useful to agriculture. In: Germ plasm resources: a symposium presented at the Chicago meeting of the American Association for the Advancement of Science, pp 3–19

Harlan JR (1968) On the origin of barley. In: Barley: origin, botany, culture, winterhardiness, genetics, utilization, pests. Agriculture Handbook No 338, pp 9–31. U.S. Department of Agriculture, Washington, DC

Harlan JR, Wet JMJD (1973) Quality of evidence for origin and dispersal of cultivated plants. Curr Anthropol 14:51–62

Helbaek H (1959) Domestication of food plants in the old world. Science 130:365–372

Helbaek H (1969) Plant collecting, day-farming, and irrigation agriculture in prehistoric Deh Luran. In: Hole F, Flannery KV, Neely JA (eds) Prehistory and human ecology of the Deh Luran Plain. Michigan Museum of Anthropology, No 1, University of Michigan, Ann Arbor, pp 383–426

Hemming MN, Peacock WJ, Dennis ES, Trevaskis B (2008) Low-temperature and daylength cues are integrated to regulate FLOWERING LOCUS T in barley. Plant Physiol 147:355–366

Hillman GC, Davies MS (1990) Measured domestication rates in wild wheats and barley under primitive cultivation, and their archaeological implications. J World Prehistory 4:157–222

Hori K, Sato K, Takeda K (2007) Detection of seed dormancy QTL in multiple mapping populations derived from crosses involving novel barley germplasm. Theor Appl Genet 115:869–876

Jakob S, Blattner F (2006) A chloroplast genealogy of *Hordeum* (poaceae): Long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference. Mol Biol Evol 23:1602–1612

Jones H, Leigh FJ, Mackay I, Bower MA, Smith LMJ, Charles MP, Jones G, Jones MK, Brown TA, Wayne P (2008) Population-based resequencing reveals that the flowering time adaptation of cultivated barley originated east of the Fertile Crescent. Mol Biol Evol 25:2211–2219

Kandemir N, Kudrna D, Ulrich S, Kleinhofs A (2000) Molecular marker assisted genetic analysis of head shattering in six-rowed barley. Theor Appl Genet 101:203–210

Karsai I, Szucs P, Koszegi B, Hayes PM, Casas A, Bedo Z, Veisz O (2008) Effects of photo and thermo cycles on flowering time in barley: a genetical phenomics approach. J Exp Bot 59:2707–2715

Kikuchi R, Kawahigashi H, Ando T, Tonooka T, Handa H (2009) Molecular and functional characterization of PEBP genes in barley reveal the diversification of their roles in flowering. Plant Physiol 149:1341–1353

Kimura S, Sakurai N, Itoh T (1999) Different distribution of cellulose synthesizing complexes in brittle and non-brittle strains of barley. Plant Cell Physiol 40:335–338

Kislev ME, Nadel D, Carmi I (1992) Epipalaeolithic (19,000 Bp) cereal and fruit diet at Ohalo-Ii, Sea of Galilee, Israel. Rev Palaeobot Palynol 73:161–166

Komatsuda T, Mano Y (2002) Molecular mapping of the intermedium spike-c (int-c) and non-brittle rachis 1 (btr1) loci in barley (Hordeum vulgare L.). Theor Appl Genet 105:85–90

Komatsuda T, Salomon B, Bryngelsson T, Rv B (2001) Phylogenetic analysis of *Hordeum marinum* Huds. based on nucleotide sequences linked to the *vrs1* locus. Plant Syst Evol 227:137–144

Komatsuda T, Maxim P, Senthil N, Mano Y (2004) High-density AFLP map of nonbrittle rachis 1 (btr1) and 2 (btr2) genes in barley (Hordeum vulgare L.). Theor Appl Genet 109:986–995

Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H et al (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci U S A 104:1424–1429

Komatsuda T, Salomon B, von Bothmer R (2009) Evolutionary process of Hordeum brachyantherum 6x and related tetraploid species revealed by nuclear DNA sequences. Breed Sci 59:611–616

Konishi S, Izawa T, Lin S, Ebana K, Fukuta Y, Sasaki T, Yano M (2006) An SNP caused loss of seed shattering during rice domestication. Science 213:1392–1996

Laurie D (1997) Comparative genetics of flowering time. Plant Mol Biol 35:167–177

Laurie D, Pratchett N, Bezant J, Snape J (1994) Genetic analysis of a photoperiod response gene on the short arm of chromosome 2(2H) of Hordeum vulgare (barley). Heredity 72:619–627

Laurie D, Pratchett N, Bezant J, Snape J (1995) RFLP mapping of five major genes and eight quantitative trait loci controlling time in a winter x spring barley (Hordeum vulgare L.) cross. Genome 38:575–585

Leonard WH (1942) Inheritance of fertility in the lateral spikelets of barley. Genetics 27:299–316

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F (2007) Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. J Exp Bot 58:425–437

Leymarie J, Robayo-Romero ME, Gendreau E, Benech-Arnold RL, Corbineau F (2008) Involvement of ABA in induction of secondary dormancy in barley (Hordeum vulgare L.) seeds. Plant Cell Physiol 49:1830–1838

Li W, Gill B (2006) Multiple genetic pathways for seed shattering in the grasses. Funct Integr Genomics 6:300–309

Li C, Ni P, Francki M, Hunter A, Zhang Y et al (2004) Genes controlling seed dormancy and pre-harvest sprouting in a rice-wheat-barley comparison. Funct Inter Genomics 2004:84–93

Liu C, Atkinson M, Chinoy C, Devos K, Gale M (1992) Nonhomoeologous translocations between group 4, 5 and 7 chromosomes within wheat and rye. Theor Appl Genet 83:305–312

Lundqvist U (1991) Coordinator's report: ear morphology genes. Barley Genet Newsl 20:85–86

Lundqvist U, Lundqvist A (1988) Induced intermedium mutant in barley: origin, morphology and inheritance. Hereditas 108:13–26

Lundqvist U, Franckowiak JD, Konishi T (1997) New and revised descriptions of barley genes. Barley Genet Newsl 26:22–516

Matsumoto K, Teramura T, Tabushi J (1963) Development analysis of the rachis desarticulation in Triticum. Wheat Inf Serv 15–16:23

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F (2006) Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8'-hydroxylase. Plant J 45:942–954

Morrell PL, Clegg MT (2007) Genetic evidence for a second domestication of barley (Hordeum vulgare) east of the Fertile Crescent. Proc Natl Acad Sci U S A 104:3289–3294

Muller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W (2001) In vitro interactions between barley TALE homeodomain proteins suggest a role for protein-protein associations in the regulation of Knox gene function. Plant J 27:13–23

Murai K, Miyamae M, Kato H, Takumi S, Ogihara Y (2003) WAP1, a wheat APETALA1 homolog, plays a central role in the phase transition from vegetative to reproductive growth. Plant Cell Physiol 44:1255–1265

Nalam V, Vales M, Watson C, Kianian S, Riera-Lizarazu O (2006) Map-based analysis of genes affecting the brittle rachis character in tetraploid wheat (*Triticum turgidum* L.). Theor Appl Genet 112:373–381

Nevo E, Chen GX (2010) Drought and salt tolerances in wild relatives for wheat and barley improvement. Plant Cell Environ 33:670–685

Nishikawa T, Salomon B, Komatsuda T, Bothmer R, Kadowaki K (2002) Molecular phylogeny of the genus Hordeum using three chloroplast DNA sequences. Genome 45:1157–1166

Oliver SN, Finnegan EJ, Dennis ES, Peacock WJ, Trevaskis B (2009) Vernalization-induced flowering in cereals is associated with changes in histone methylation at the VERNALIZATION1 gene. Proc Natl Acad Sci U S A 106:8386–8391

Orabi J, Backes G, Wolday A, Yahyaoui A, Jahoor A (2007) The Horn of Africa as a centre of barley diversification and a potential domestication site. Theor Appl Genet 114:1117–1127

Petersen G, Seberg O (2004) On the origin of the tetraploid species Hordeum capense and H-secalinum (Poaceae). Syst Bot 29:862–873

Pourkheirandish M, Wicker T, Stein N, Fujimura T, Komatsuda T (2007) Analysis of the barley chromosome 2 region containing the six-rowed spike gene vrs1 reveals a breakdown of rice-barley micro collinearity by a transposition. Theor Appl Genet 114:1357–1365

Prada D, Ullrich S, Molina-Cano J, Cistue L, Clancy J, Romagosa I (2004) Genetic control of dormancy in a Triumph/Morex cross in barley. Theor Appl Genet 109:62–70

Ramsay L, Comadran J, Druka A, Marshall DF, Thomas WT et al (2011) INTERMEDIUM-C, a modifier of lateral spikelet fertility in barley, is an ortholog of the maize domestication gene TEOSINTE BRANCHED 1. Nat Genet 43:169–172

Romagosa I, Han F, Clancy JA, Ullrich SE (1999) Individual locus effects on dormancy during seed development and after ripening in Barley. Crop Sci 39:74–79

Saisho D, Purugganan MD (2007) Molecular phylogeography of domesticated barley traces expansion of agriculture in the old world. Genetics 177:1765–1776

Saisho D, Pourkheirandish M, Kanamori H, Matsumoto T, Komatsuda T (2009) Allelic variation of row type gene Vrs1 in barley and implication of the functional divergence. Breed Sci 59:621–628

Sakuma S, Pourkheirandish M, Matsumoto T, Koba T, Komatsuda T (2010) Duplication of a well-conserved homeodomain-leucine zipper transcription factor gene in barley generates a copy with more specific functions. Funct Integr Genomics 10:123–133

Sakuma S, Salomon B, Komatsuda T (2011) The domestication syndrome genes responsible for the major changes in plant form in the Triticeae crops. Plant Cell Physiol 52:738–749

Salamini F, Özkan H, Brandolini A, Schäfer-Pregl R, Martin W (2002) Genetics and geography of wild cereal domestication in the Near East. Nat Rev Genet 3:429–441

Sameri M, Takeda K, Komatsuda T (2006) Quantitative trait loci controlling agronomic traits in recombinant inbred lines from a cross of oriental- and occidental-type barley cultivars. Breed Sci 56:243–252

Sato K, Matsumoto T, Ooe N, Takeda K (2009) Genetic analysis of seed dormancy QTL in barley. Breed Sci 59:645–650

Schmalenbach I, March TJ, Bringezu T, Waugh R, Pillen K (2011) High-resolution genotyping of wild barley introgression lines and fine-mapping of the threshability locus thresh-1 using the Illumina GoldenGate assay. G3 (Bethesda) 1:187–196

Senthil N, Komatsuda T (2005) Inter-subspecific maps of non-brittle rachis genes btr1/btr2 using occidental, oriental and wild barley lines. Euphytica 145:215–220

Shakhatreh Y, Haddad N, Alrababah M, Grando S, Ceccarelli S (2010) Phenotypic diversity in wild barley (Hordeum vulgare L. ssp spontaneum (C. Koch) Thell.) accessions collected in Jordan. Genet Resour Crop Evol 57:131–146

Simons K, Fellers J, Trick H, Zhang Z, Tai Y-S, Gill B, Faris J (2006) Molecular characterization of the major wheat domestication gene Q. Genetics 172:547–555

Stein N, Prasad M, Scholz U, Thiel T, Zhang H, Wolf M, Kota R, Varshney RK, Perovic D, Grosse I, Graner A (2007) A 1,000-loci transcript map of the barley genome: new anchoring points for integrative grass genomics. Theor Appl Genet 114:823–839

Sun G, Pourkheirandish M, Komatsuda T (2009) Molecular evolution and phylogeny of the RPB2 gene in the genus Hordeum. Ann Bot 103:975–983

Szucs P, Karsai I, von Zitzewitz J, Meszaros K, Cooper LL, Gu YQ, Chen TH, Hayes PM, Skinner JS (2006) Positional relationships between photoperiod response QTL and photoreceptor and vernalization genes in barley. Theor Appl Genet 112:1277–1285

Takahashi R (1955) The origin and evolution of cultivated barley. In: Demerec M (ed) Advances in genetics. Academic, New York, NY, pp 227–266

Takahashi R, Hayashi J (1964) Linkage study of two complementary genes for brittle rachis in barley. Ber Ohara Inst Landwirtsch Biol Okayama Univ 12:99–105

Taketa S, Amano S, Tsujino Y, Sato T, Saisho D et al (2008) Barley grain with adhering hulls is controlled by an ERF family transcription factor gene regulating a lipid biosynthesis pathway. Proc Natl Acad Sci U S A 105:4062–4067

Tanno K, Willcox G (2006) How fast was wild wheat domesticated? Science 311:1886

Tanno K, Willcox G (2012) Distinguishing wild and domestic wheat and barley spikelets from early Holocene sites in the Near East. Veget Hist Archaeobot 21:107–115

Taoka K, Ohki I, Tsuji H, Furuita K, Hayashi K et al (2011) 14-3-3 proteins act as intracellular receptors for rice Hd3a florigen. Nature 476:332–335

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES (2006) HvVRN2 responds to daylength, whereas HvVRN1 is regulated by vernalization and developmental status. Plant Physiol 140:1397–1405

Turner A, Beales J, Faure S, Dunford R, Laurie D (2005) The pseudo-response regulator Ppd-H1 provides adaptation to photoperiod in barley. Science 310:1031–1034

Ubisch G (1915) Analyse eines Falles von Bastardatavismus und Faktorenkoppelung bei Gerste. Zeits Indukt Abstammungs Vererbungsl 14:226–237

Ullrich SE, Lee H, Clancy JA, del Blanco IA, Jitkov VA, Kleinhofs A, Han F, Prada D, Romagosa I, Molina-Cano JL (2009) Genetic relationships between preharvest sprouting and dormancy in barley. Euphytica 168:331–345

Wang R-C, Bothmer Rv, Dvorak J, Fedak G, Linde-Laursen I, Muramatsu, M. (1994) Genome symbols in the Triticeae (Poaceae). In: Wang R-C, Jensen K, Jaussi C (eds) Proceedings of the 2nd international Triticeae symposium, Logan, Utah, pp 29–34

Woodward RW (1947) The Ih, I, i allels in Hordeum deficiens genotypes of barley. J Am Soc Agron 39:474–482

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene VRN1. Proc Natl Acad Sci U S A 100:6263–6268

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen J, Eckenique V, Dubcovsky J (2004) The wheat VRN2 gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1644

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene VRN3 is an orthologue of FT. Proc Natl Acad Sci U S A 103:19581–19586

Yasuda S (1969) Linkage and pleiotropic effects on agronomic characters of the genes for spring growth habit. Barley Newsl 12:57–58

Youssef H, Koppolu R, Schnurbusch T (2012) Re-sequencing of vrs1 and int-c loci shows that labile barleys (Hordeum vulgare convar. labile) have a six-rowed genetic background. Genet Resour Crop Evol 59:1319–1328

Zhang FC, Gutterman Y (2003) The trade-off between breaking of dormancy of caryopses and revival ability of young seedlings of wild barley (Hordeum spontaneum). Can J Bot (Revue Canadienne De Botanique) 81:375–382

Zhang H, Reader SM, Liu X, Jia JZ, Gale MD, Devos KM (2001) Comparative genetic analysis of the Aegilops longissima and Ae. sharonensis genomes with common wheat. Theor Appl Genet 103:518–525

Zhang F, Chen G, Huang Q, Orion O, Krugman T, Fahima T, Korol AB, Nevo E, Gutterman Y (2005) Genetic basis of barley caryopsis dormancy and seedling desiccation tolerance at the germination stage. Theor Appl Genet 110:445–453

Zhang XQ, Li CD, Panozzo J, Westcott S, Zhang GP, Tay A, Appels R, Jones M, Lance R (2011) Dissecting the telomere region of barley chromosome 5HL using rice genomic sequences as references: new markers for tracking a complex region in breeding. Mol Breed 27:1–9

Zitzewitz J, Szucs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen THH, Hayes PM, Skinner JS (2005) Molecular and structural characterization of barley vernalization genes. Plant Mol Biol 59:449–467

Zohary D (1964) Spontaneous brittle six-row barleys, their nature and origin. In: Barley genetics, I: Proceedings of 1st international barley genetics symposium, 26–31 Aug 1963, Wageningen, pp 27–31

Zohary D, Hopf M (2000) Domestication of plants in the old world. Oxford University Press, New York, NY

Chapter 4
Shoot and Inflorescence Architecture

Laura Rossini, Ron Okagaki, Arnis Druka, and Gary J. Muehlbauer

4.1 Introduction

Shoot and inflorescence architecture are morphological structures that impact crop productivity. An understanding of the developmental processes that control these structures has been advanced by the identification and characterization of numerous mutant and natural alleles. Recent advances in barley genomics have facilitated the isolation of these genes. The aims of this chapter are to describe the developmental processes controlling shoot and inflorescence development, the key genes regulating these processes, and the potential approaches to exploit this information for barley improvement. To that end, we have divided this chapter into three major sections including a basic description of barley shoot and inflorescence architecture, genetic control of shoot and inflorescence architecture, and potential approaches to alter shoot and inflorescence architecture that may result in increased crop productivity.

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4.2 Shoot and Inflorescence Development

4.2.1 The Embryo and the Shoot Apical Meristem

A mature grass caryopsis contains a highly organized embryo consisting of different regions with specialized functions (MacLeod and Palmer 1966):

1. The scutellum, a structure unique to grass species that mediates release of hydrolytic enzymes and subsequently the transfer of nutrients from the endosperm during germination. The scutellum has been interpreted as a modified cotyledon (Rudall et al. 2005 and references therein).
2. The radicle with the root apical meristem protected by the coleorhiza.
3. The epicotyl comprising the shoot apical meristem (SAM) and leaf primordia enclosed by the coleoptile.
4. The nodal region between the epicotyl and the radicle.

The architecture of the aerial part of a plant is ultimately determined by the activity of the SAM. Thus, organization of the plant body starts during embryogenesis when the SAM is formed (Fig. 4.1). Details on the development of the barley embryo can be found in a recent review (Gubatz and Shewry 2011).

Based on histological analyses, the SAM can be seen as organized in layers and zones, reflecting different rates and orientation of cell divisions, functions, and developmental fates (Bowman and Eshed 2000; Brand et al. 2001). Meristem cells belong to clonally distinct layers: the outer tunica and the inner corpus. The tunica is characterized by anticlinal cell division: newly formed cell walls are oriented perpendicular to the meristem surface, and daughter cells remain in the same layer as the mother cell. Cell division in the corpus can occur in different planes. The barley SAM, similar to maize, appears to be structured in one-layered tunica (L1) and corpus (L2), although a three-layer organization cannot be excluded (Döring et al. 1999). In dicots and many grasses, the tunica is structured into an

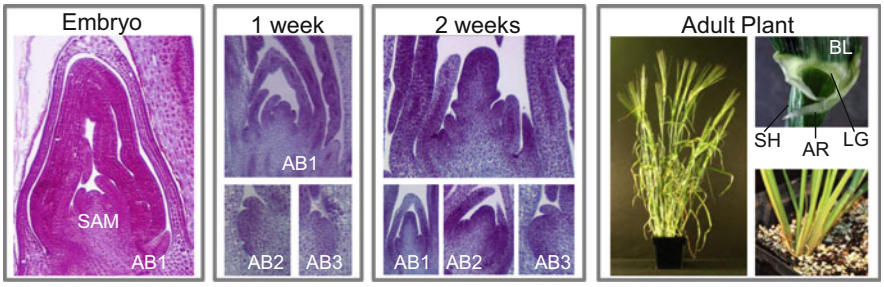

Fig. 4.1 Vegetative development. Histological longitudinal sections of the shoot apical meristem (SAM) and axillary buds (AB) are shown of the embryo, 1-week- and 2-week-old seedlings. An adult plant and seedling leaf and tillers are also shown. *BL* leaf blade, *SH* leaf sheath, *LG* ligule, *AR* auricle

epidermal L1 layer and a subepidermal L2 layer (Barnard 1964; Clark and Fisher 1988).

Intersecting these layers, three zones can be distinguished to describe the radial patterning of the SAM:

1. The peripheral zone is characterized by high cell division rates and is the site of lateral organ formation.
2. The central zone hosts a reservoir of slowly dividing pluripotent stem cells responsible for the maintenance of the meristem.
3. The rib zone originates stem tissues.

In monocots, the central tissues of the stem derive from the primary thickening meristem located below the apical meristem (Leyser and Day 2003). In barley, the activity of the SAM begins during embryogenesis with the production of 3–4 leaf primordia. A lateral bud is present in the axil of the coleoptile and often in the axil of the first true leaf (Fig. 4.1; Kirby and Appleyard 1987). Embryogenesis is complete when the caryopsis is fully mature and desiccated (Bossinger et al. 1992a).

4.2.2 Shoot Development

After germination, shoot growth depends on the activity of the SAM that produces reiterative modules called phytomers. The basic phytomer consists of an internode (stem segment), a leaf, and an axillary bud (Weatherwax 1923; Sharman 1942). Variations of this basic form can model other types of organs (e.g., floral structures; see below).

Barley is characterized by a distichous arrangement of leaves and spikelets on vegetative stems (main culm and tillers) and inflorescence axes (rachis), respectively. Leaves are strap shaped with parallel veins and a prominent midrib. The distal lamina (leaf blade) and proximal sheath are separated by the ligule, which is flanked by two ear-like projections called auricles (Fig. 4.1).

In grasses, leaves are initiated by recruitment of a ring of founder cells undergoing periclinal cell divisions in the peripheral zone of the meristem (Bossinger et al. 1992b). This region corresponds to the disk of leaf insertion where the internode is also organized in a basipetal position (Sharman 1942). Different meristematic cell layers participate in leaf development in grasses with one- or two-layered tunica (reviewed in Bossinger et al. 1992a). In barley, the L1 (tunica) originates the epidermal layer and part of the mesophyll, and the L2 (corpus) gives rise to all other parts of the plant (Döring et al. 1999). With the development of the midrib in the ring-shaped leaf primordium, the centrolateral axis of symmetry and dorsoventral (or abaxial-adaxial) polarity are established. With progression of proximodistal differentiation, the developing leaf becomes hood shaped enclosing the shoot apex and younger primordia. Leaf growth is the result of coordinated cell division and expansion patterns along the primordium known as a basipetal wave.

Characteristic of the basipetal wave is progressive cessation of the cell division from the leaf tip downward. As a result, when the blade is fully developed, cells are still dividing at the base of the sheath.

Development of the vegetative internode involves both radial and vertical growth. The latter is mediated by a specialized intercalary meristem located at the base of the internode itself (Bossinger et al. 1992a). Depending on genotype and time of sowing, a variable number of leaves can be formed on a barley culm with a number of basal internodes that remain short and others that elongate following transition from the vegetative to reproductive phase (Kirby and Appleyard 1987). After the nodes and the apical regions of elongating internodes have completed their development, intercalary meristems continue their activity causing stem growth and pushing the developing spike upwards (Bossinger et al. 1992a, see below). The last vegetative leaf formed on the culm is called the flag leaf, and the sheath of the flag leaf encloses the spike.

In barley and other grasses, lateral shoots called tillers develop from vegetative axillary meristems (AXMs) present in the axils of leaves at the base of the plant (crown) (Fig. 4.1; McSteen and Leyser 2005). Axillary buds form at basal internodes and consist of an AXM with young leaf primordial enclosed by the prophyll, a two-keeled leaflike organ that may be seen as the first leaf of the lateral shoot (Bossinger et al. 1992b). Tiller development involves three main developmental stages (Schmitz and Theres 2005). During the first stage, the AXM is initiated, which results in a stem cell population located in a leaf axil. The second stage balances cell division in the AXM with differentiation of cells into primordial leaves and produces an axillary bud. Finally, outgrowth of the axillary bud forms a tiller. Tillers can develop inflorescence meristems (see below), resulting in seed development. Although not characterized in barley, outgrowth is likely regulated by interactions of the environment and at least three plant hormones, auxin, cytokinin, and strigolactone (reviewed in Domagalska and Leyser 2011). In barley, AXM derives through cell divisions from more than one meristem layer so that the layers of the AXM derive directly from the same layers in the main shoot (Bossinger et al. 1992b; Döring et al. 1999). The AXM later acquires the typical structure of the SAM initiating organogenesis in a similar manner. Primary tillers formed by the main culm can produce secondary tillers, which in turn may produce tertiary tillers in a reiterative pattern, defining the architecture of the plant. In general, only a subset of AXMs generate tillers, while the rest are arrested at the bud stage. The final number of stems varies considerably depending on genetic and environmental factors, with more tillers generally produced by winter compared to spring cultivars (Kirby and Appleyard 1987).

4.2.3 Inflorescence Development

The mature barley inflorescence, called spike, head, or ear, consists of the floral stem or rachis and floral units called spikelets. Spikelets consist of a floret and two

subtending bracts called empty or outer glumes. Each rachis node bears three spikelets. In wild barley and two-rowed cultivars, only the central spikelet is fertile, while the lateral spikelets are sterile and remain underdeveloped. In six-rowed barley cultivars and mutants, all three florets mature to produce grains (Kirby and Appleyard 1987; Komatsuda et al. 2007).

Transition from the vegetative to the reproductive phase in spring barley grown under favorable temperature and light conditions typically occurs during early stages of seedling development (2–4 weeks after germination). In general, phase transition is under control of genetic, hormonal, and environmental factors. The double ridge stage marks the transition of the SAM to an inflorescence meristem (Fig. 4.2; Kirby and Appleyard 1987). At this stage, the apex is elongated about 1 mm in length, and each "double ridge" corresponds to a pair constituted by a leaf primordium and a lateral meristem (Kirby and Appleyard 1987). As inflorescence development progresses, leaf initials fail to develop, while lateral meristems become the main growing points that will originate spikelet triplet meristems (STM) that in turn will develop in three spikelet meristems (SM; Bossinger et al. 1992a), one central and two lateral. This is characteristic of the so-called triple mound stage (Fig. 4.2). Each SM gives rise to a floral meristem (FM). Two outer glume primordia (OGP) originate at the base of each FM. OGPs develop into outer glumes, and FMs give rise to floral organ primordia. Next, the glume-, lemma-, and stamen-primordium stages undergo sequential differentiation to

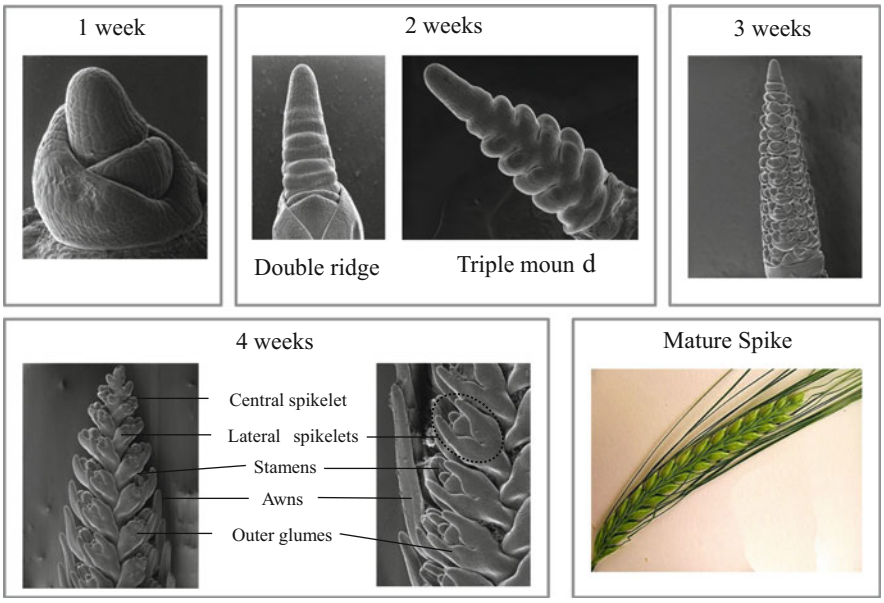

Fig. 4.2 Inflorescence development. Inflorescence development in a two-rowed genotype. Development is shown from the shoot apical meristem (SAM) at 1-week- to 4-week-old inflorescences (stamen primordium stage). A mature two-rowed spike is also pictured

mature spikelet structures (Fig. 4.2). A detailed description of the histogenesis and sequence of floral organ differentiation can be found in Bossinger et al. (1992a). Spikelet differentiation is not synchronous along the immature inflorescence axis—spikelets in the central region develop earlier than basal and especially apical spikelets. The apex continues to initiate new SMs until the awn primordium stage, when the final number of spikelet primordia is defined and the layout of spike and spikelet structures is completed (Kirby and Appleyard 1987). After this stage, the spike undergoes growth and further differentiation followed by fertilization, caryopsis development, and grain filling. These processes altogether determine the number and size of grains produced per spike (reviewed in Sreenivasulu and Schnurbusch 2012).

Development of central and lateral spikelets is not synchronous. Development of the lateral spikelets is slower, eventually terminating altogether resulting in very rudimentary and sterile structures that together, with fully developed, fertile central spikelets, form barley inflorescence called a "two-rowed spike." This is the predominant spikelet developmental pattern occurring in the wild and most cultivated barleys. Recessive mutations in *Vrs1* genes can promote development of the lateral spikelets, so that in some cases, later in development, lateral spikelets "catch up" with central ones and become morphologically indistinguishable developing into the inflorescence called a "six-rowed spike" (Kirby and Appleyard 1987; Komatsuda et al. 2007).

Apart from outer glumes, the spikelet axis (or rachilla) bears the lemma, an abaxial floral bract that encloses a single floret and carries a bristlelike distal appendage called the awn. The lemma-awn complex has been interpreted as a reduced vegetative leaf with the awn and lemma corresponding to the leaf blade and sheath, respectively (Dahlgren et al. 1985; Clifford 1988; Pozzi et al. 2000). The floret develops in the lemma axil and comprises a two-keeled palea, two lodicules, three stamens, and the pistil. The palea is the first organ formed on the floret axis, occupies an adaxial position, and is considered homologous to the prophyll (Williams 1975). The presence of two midribs has been interpreted as a distinctive feature of all foliar organs forming after a branching point (Bossinger et al. 1992a). Although barley is predominantly an autogamous species, lodicules can swell up pushing apart the palea and lemma and allowing anther exertion and cross-pollination (Nair et al. 2010).

4.2.4 *Phytomeric Models for Interpretation of Barley Shoot and Inflorescence Architecture*

Bossinger et al. (1992a, b) proposed a phytomeric interpretation of barley development, integrating information from anatomical, histological, and genetic analyses of wild-type and mutant plants and providing a general model for the organization of both vegetative and reproductive structures. Two distinct phytomer types were defined:

- The type I phytomer essentially coincides with the classical phytomer according to Weatherwax (1923) and Sharman (1942); it is present in vegetative parts of the plant, and with some modifications (e.g., suppression of leaf development), it is also found in the inflorescence.
- The type 2 phytomer is a special structure present at branching points. This type of phytomer is exemplified by the prophyll and can be used to model other two-keeled organs (e.g., coleoptile, palea) that are interpreted as deriving from the fusion of two leaves (Bossinger et al. 1992b).

Based on this interpretation, the mature barley plant is organized in six developmental regions, and specific phytomers are present in each region (Bossinger et al. 1992a).

Forster et al. (2007) presented a revision of the Bossinger et al. (1992a) model based on a single type of phytomeric unit with individual organs representing single phytomers (e.g., leaf, lemma) or fused pairs of phytomers (e.g., palea, prophyll).

4.3 Genetic Control of Shoot and Inflorescence Architecture

Barley geneticists and breeders have collected and characterized thousands of mutants (e.g., Franckowiak and Lundqvist 2010; Lundqvist et al. 1996). Many of these mutants exhibit shoot or inflorescence phenotypes. Chromosomal positions offer a starting point for isolation of the corresponding genes through map-based and comparative genomic approaches (Pozzi et al. 2003; Rossini et al. 2006). To facilitate genetic studies, 881 of these mutant lines were introgressed into a common background, the cultivar Bowman. Recently, Druka et al. (2011) genotyped these backcross lines using 2,943 mapped SNP markers (Close et al. 2009) and positioned 426 mutant alleles to the genetic map. The SNP-based location of 426 mutant alleles provides a starting point for introgressing potentially useful genes in breeding programs and facilitates map-based cloning efforts. In this section we describe the genes regulating shoot and inflorescence architecture.

4.3.1 Genetic Control of Tiller Number

As described above, tillers are modified branches developing from vegetative AXMs located in leaf axils at the base, or crown, of the plant. Tiller number and vigor play a major role in biomass and grain yield, and therefore these traits have been the target of genetic studies and gene isolation projects. Large collections of barley mutants that impact tiller number have been identified and characterized (Fig. 4.3). They can be subdivided in four major groups. The first group includes mutants that do not tiller; they produce a single main culm. This group is defined by

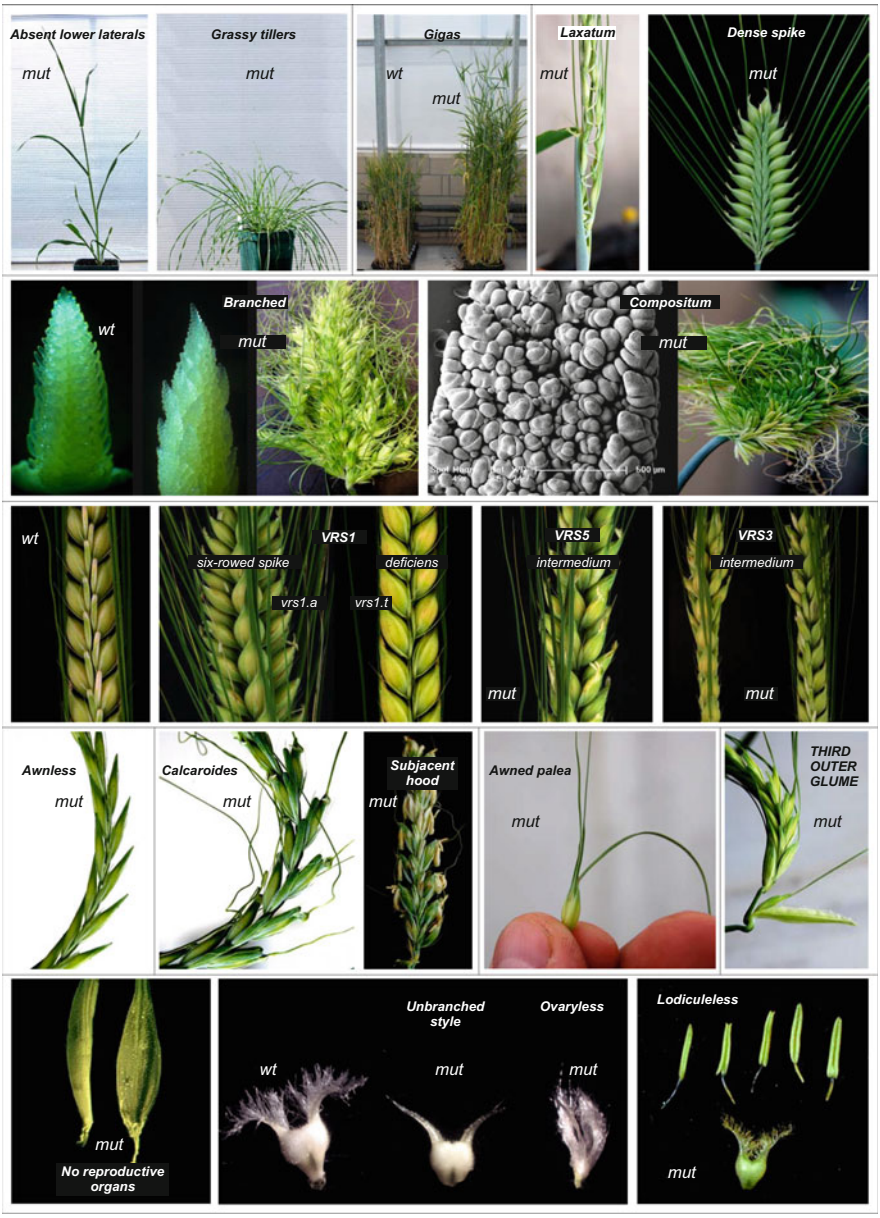

Fig. 4.3 Barley developmental mutants. Genes that have been isolated are in uppercase. Genes in lowercase have not been isolated. Abbreviations: *mut* mutant allele, *wt* wild-type allele. Developing inflorescences dissected from the *branched* and *compositum* mutants are about 1 month old

the *uniculm2* (*cul2*) mutant (Babb and Muehlbauer 2003). Mutants that exhibit a low number of tillers belong to the second group, which is defined by *low number of tillers1* (*lnt1*; Dabbert et al. 2010), *absent lower laterals1* (*als1*; Fig. 4.3; Dabbert et al. 2009), and *uniculme4* (*cul4*) mutants. The third group comprises mutants with modestly reduced tillering and can be defined by the *intermedium-b* (*int-b*) and *semibrachytic* (*uzu*) mutants. The fourth group of mutants exhibits an increased number of tillers compared to wild type and can be defined by the *granum-a* (*gra-a*), *grassy tillers* (*grassy*; Fig. 4.3) *intermedium-c* (*int-c*; Ramsay et al. 2011), *many noded dwarf1* (*mnd1*), and *many noded dwarf6* (*mnd6*) mutants. The third and fourth groups probably include many more mutants, for example, those with altered spike development, for which tillering has not been measured. Recessive alleles for each of these mutations have been introgressed into the Bowman genetic background (Druka et al. 2011).

Plants carrying recessive mutations in the *Uniculm2* (*Cul2*) gene do not tiller (Babb and Muehlbauer 2003). Histological examination of the shoot apex in mature embryos, 1-week and 2-week old *cul2* mutant plants, showed that the AXM appeared to be initiated but did not develop into an axillary bud. Double mutant analysis of the *cul2* mutant with low (*lnt1*, *als1*, *cul4*, *int-b*, and *uzu*) and high tillering mutants (*mnd1*, *mnd6*, and *gra*) all resulted in a uniculm phenotype. Taken together, these results indicate that the *Cul2* gene is a regulator of axillary bud development. Recently, two mutants that suppressed the *cul2* mutant phenotype were isolated and given the names *Suppressor of uniculm2-1 and -2* (*Scu2-1 and Scu2-2*; Bilgic, Okagaki, and Muehlbauer, unpublished results). Double homozygous mutants containing *scu2-1* or *scu2-2* and *cul2* (*scu2*/*scu2*; *cul2*/*cul2*) result in plants that exhibit tiller development. Allelism tests indicated that *scu2-1* and *scu2-2* are allelic. Finally, transcriptome analysis using the Affymetrix Barley1 GeneChip (Close et al. 2004) found increased expression of plant defense and stress-related genes in the *cul2* mutant compared to wild-type plants (Okagaki et al. 2013).

Plants carrying recessive mutations at the *Als1*, *Lnt1*, or *Cul4* loci in the Bowman genetic background typically develop 1–3 tillers, whereas wild-type Bowman exhibits approximately 13–27 tillers depending upon if it is grown in the field or greenhouse, respectively (Babb and Muehlbauer 2003). However, there is variable phenotypic expression (e.g., *cul4*) that is dependent on unknown environmental factors (Muehlbauer and Rossini, unpublished). Histological investigations of shoot apices of these mutants suggest that the lack of additional, secondary tillers in these mutants is the result of an inability of axils in the primary tillers to form axillary buds (Dabbert et al. 2009, 2010; Muehlbauer unpublished data). Double mutant analysis of this group of mutants with other high tillering mutants exhibited the low tillering mutant phenotype, indicating that the *lnt1*, *als1*, and *cul4* are epistatic to the high tillering mutants (Dabbert et al. 2009, 2010; Muehlbauer unpublished results). Transcriptome analysis of the *als1* mutant compared to wild-type controls showed that the *als1* mutant exhibited upregulation of stress-related genes. Noteworthy, even though the *lnt1* mutant exhibits a similar low tillering phenotype

compared to the *als1* mutant, stress-related genes were not upregulated in the *lnt1* mutant.

Transcriptome analysis uncovered the *JuBel2* gene that was expressed in wild type but not in the *lnt1* mutant (Dabbert et al. 2010). Mapping and sequence analysis of the *lnt1.a* allele further supported the correspondence between the *JuBel2* gene and the *Lnt1* locus. The JuBEL2 protein was annotated as a BELL-family homeodomain transcription factor (Müller et al. 2001). Homology searches indicated that *JuBEL2* is related to the rice *QTL of seed shattering in chromosome 1* (*qSH1*) gene (Konishi et al. 2006) and the *Arabidopsis BELLRINGER* (*BLR*) gene (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003). In *Arabidopsis*, loss of function alleles in *BLR* results in a variety of phenotypes (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003).

The specific function of the barley JuBEL2 protein is not known. However, studies in *Arabidopsis* provide some insight. In Arabidopsis, the *BLR* protein binds the *SHOOT MERISTEMLESS* (*STM*) protein and helps transport it to the nucleus (Cole et al. 2006; Rutjens et al. 2009). *STM* is required for shoot apical and axillary meristem development (Long and Barton 2000), and *Arabidopsis* plants carrying mutations in *BLR* and two related *BELL-like* genes, *POUND-FOOLISH* and *ARABIDOPSIS THALIANA HOMEOBOX GENE 1*, lack the SAM, providing evidence that *STM* function requires BELL-like proteins (Rutjens et al. 2009). The barley JuBEL2 protein has been shown to bind to class I KNOX proteins including Hooded/BKN3, which is a barley homologue of *STM* (Müller et al. 2001). Thus, it is likely that *JuBel2* interacts with *STM*-like genes in barley and promotes both shoot and axillary meristem development and tiller development.

Recessive mutations in the *Int-b* and *Uzu* genes modestly reduce tiller number. Plants carrying the *int-b* or *uzu* mutation in the Bowman genetic background develop an average of 12 and 16 tillers, respectively (Babb and Muehlbauer 2003). The *uzu* gene is an important agronomic dwarfing gene (see below for more details), and it encodes a putative brassinosteroid receptor (Chono et al. 2003). Axillary meristem development in *int-b* or *uzu* mutants has not been studied, and it is not known if the reduction in tillering is associated with altered axillary meristem or axillary bud development.

Plants carrying recessive mutations in the *Gra-a*, *Mnd1*, and *Mnd6* genes are short and develop 2–3 times the number of tillers compared to wild-type controls (Babb and Muehlbauer 2003). As expected, the *gra-a* mutant exhibits an increased number of AXMs and axillary buds. Noteworthy, *gra-a* occasionally exhibits two shoot apical meristems (Babb and Muehlbauer 2003). Examination of the AXMs in the *mnd1* and *mnd6* mutants has not been performed. Although the genes underlying these three mutations have not been isolated, these mutants resemble the rice *dwarf3*, *dwarf10*, and *high-tillering dwarf1* mutants that encode orthologs of the *Arabidopsis MAX* genes involved in the strigolactone pathway (Ishikawa et al. 2005; Zou et al. 2006; Arite et al. 2007). Consistent with the rice and *Arabidopsis* mutants, it is likely that the *gra-a*, *mnd1*, and *mnd6* mutants promote the development of tillers.

Quantitative trait loci (QTL) associated with tiller number have been mapped in barley. In general, the amount of variation explained by each QTL was small. Gyenis et al. (2007) used a wild barley introgression population and mapped three QTL for tiller number on chromosomes 2H bin 3, 5H bin 6–8, and 6H bin 10–11. The range of variation explained by each QTL was between 10.3 and 15.7 %. Baum et al. (2003) also found QTL for tiller number on chromosomes 2H bin 3 and 5H bin 6–8. A major tiller number QTL coinciding with the row type gene *VRS1* (see below) has been mapped in a recombinant inbred line population derived by crossing low-tillering six-rowed barley cultivar Morex to a high-tillering two-rowed cultivar Golden Promise, demonstrating that the recessive allele *vrs1.a* can reduce tillering (Druka et al. unpublished). In contrast, recessive alleles of the row type gene *VRS5* (or *INT-c*) seem to promote tillering (Ramsay et al. 2011). Together these data suggest that different row type genes may have contrasting or similar effects on reproductive and vegetative development.

4.3.2 Genetic Control of Plant Height

Plant height has received a considerable amount of attention due to the success of semidwarf varieties in other crops. For example, short stature wheat and rice crops have dramatically increased yield due to the repartitioning of assimilate from stems to grain production (Khush 2001) at the same time reducing lodging. Numerous loci have been identified that impact these traits in mapping studies, and some of these loci are introgressed into elite barley lines and are components of variety development programs.

Multiple loci controlling height have been identified including *breviaristatum* (*ari*), *brachytic* (*br*), *curly dwarf* (*cud*), *denso dwarf* (*denso*), *erectoides* (*ert*), *lazy dwarf* (*lzd*), *many noded dwarf* (*mnd*), *narrow leaf dwarf* (*nld*), *semidwarf* (*sdw*), *slender dwarf* (*sld*), *single internode dwarf* (*sid*), *semibrachytic* (*uzu*), *short culm 1* (*hcm1*), and *vegetative dwarf* (*dwf*; Swenson and Wells 1944; Zhang and Zhang 2003; Sears et al. 1981; Franckowiak 1987; Franckowiak and Pecio 1992). Many of the dwarf genes in this set have not been used in breeding programs due to deleterious pleiotropic effects resulting in low vigor and reduced grain yield. However, semidwarf varieties have been successfully used in breeding programs. The semidwarf gene *uzu* has been mapped to chromosome 3HL and has been used extensively in barley breeding programs in Asia (Hoskins and Poehlman 1971; Tsuchiya 1976; Zhang 1994, 2000; Saisho et al. 2004). Two independent mutations, referred to as *sdw1* and *denso*, have been the subject of a considerable amount of study. Both mutations map to the same location on chromosome 3HL and are thought to be allelic (Hellewell et al. 2000; Jia et al. 2009). The *sdw1* and *denso* mutations have been widely used in barley breeding in Europe, North America, and Australia (Hellewell et al. 2000). However, malting varieties carrying the *sdw1* gene have not been approved in North America. Jia et al. (2009) proposed that GA-20 oxidase was a candidate for the *sdw1/denso* gene. The *ari*-e gene, located on

chromosome 5HL, is used in European varieties (Thomas et al. 1984), and the semidwarf gene *short culm* (*hcm1*) has been proposed to be the allele used to reduce plant height in upper Midwestern US malting barley varieties (Franckowiak 2000).

Recently, the *Breviaristatum-d* (*ari-d*) gene was shown to encode a SHORT INTERNODES (SHI) family transcription factor characterized by a zinc-binding RING-finger motif and an IGGH domain (You et al. 2012). The *ari-d* mutation causes plant height reduction by approximately 50 %. The gene appears to be allelic to the *short awn2* (*lks2*) and *unbranched style4* (*ubs4*) mutations (see below).

Several other loci controlling height have been genetically characterized but not used in barley breeding. Three classes of recessive dwarf mutants referred to as gibberellic acid response mutants have been identified and include: *GA-responsive dwarf* (*grd*), *GA sensitivity* (*gse*), and *elongation* (*elo*; Chandler and Robertson 1999). Dominant mutations in the *Slender1* (*Sln1*) locus result in a dwarf phenotype due to gibberellic acid insensitivity (Chandler et al. 2002). Noteworthy, recessive mutations at the *Sln1* locus result in spindly plants. Isolation of the *Sln1* gene revealed that it encodes a DELLA protein similar to the proteins encoded by the wheat *Rht*, *Arabidopsis GAI/RGA*, and maize *D8* genes (Peng et al. 1999; Chandler et al. 2002). The wheat *Rht* gene is used in high yielding semidwarf varieties (Peng et al. 1999). In addition, the GA3-insensitive genes *Rht-H1* and *Dwf2* were mapped to the centromeric region of 2H and 2HL, respectively. Additional height genes have been identified in landraces and germplasm collections, but these alleles have not been genetically characterized or used in breeding programs (Mickelson and Rasmusson 1994; Zhang and Zhang 2003).

Due to the applied importance, most of the height mutant studies have focused on analysis of mutations that reduce plant height, ignoring those with an opposite effect. Recessive mutations in the *Gigas2* gene result in considerably increased plant height (Fig. 4.3; Franckowiak and Lundqvist 2002). The identity of the *Gigas2* gene is not known, but once isolated the gene may be potentially useful to support recent breeding efforts to enhance biomass production in barley and other species. Recently, a suppressor screen using dwarf wheat and barley mutants led to isolation of the "overgrowth" suppressor mutations. Genetic analysis of these suppressors identified novel mutations in the GA genes *Slender1* (*Sln1*) and *Spindly1* (*Spy1*; Chandler and Harding 2013).

In contrast to the single genes that have been genetically mapped and utilized in barley breeding worldwide, identifying natural variation for height is another approach to manipulating height. QTL that are associated with plant height have been identified in many mapping populations, including one of the first barley reference mapping populations derived from the Steptoe x Morex cross (Kleinhofs et al. 1993). Plant height along with many other legacy and novel plant developmental, disease resistance, and mRNA abundance traits has been mapped in this population (Druka et al. 2008). Gyenis et al. (2007) used a BC_2 population derived from a cross between the wild barley OUH602 and the elite two-rowed malting variety Harrington and identified four QTL on chromosome 1H, 2H, 3H, and 7H. These QTL explained between 11.2 and 16.1 % of the genetic variation for the trait. Although it is difficult to compare genetic maps from different mapping

populations due to different marker types used to develop the genetic map, these QTL appear to have been identified in many previous studies (e.g., Hayes et al. 1993; Marquez-Cedillo et al. 2001; Qi et al. 1998; Borem et al. 1999; de la Peña et al. 1999).

4.3.3 Genetic Control of Inflorescence Architecture

Due to implications on grain number and yield, the genetic dissection of inflorescence architecture has received much attention in cereals, and a number of genes controlling these traits have been identified in rice and maize (reviewed in Sreenivasulu and Schnurbusch 2012). Dozens of barley mutants altered in spike and spikelet morphology have been described and mapped, providing an ideal starting point for the genetic analysis of inflorescence development in the *Triticeae* (Fig. 4.3; Druka et al. 2011; Franckowiak and Lundqvist 2010; Sreenivasulu and Schnurbusch 2012; http://ace.untamo.net/bgs/). However, only few of the corresponding genes have been isolated and functionally characterized. In this section, we will focus mainly on these as they offer insight into the molecular mechanisms associated with domestication traits and developmental programs.

4.3.3.1 Inflorescence Formation

The spike is absent or very rudimentary in plants carrying the recessive *aborted spike1* (*asp1*) allele, while other plant traits are not visibly affected, indicating this gene is strictly required for inflorescence meristem development but not for vegetative development (Franckowiak et al. 2005). Inflorescence meristem activity may also depend on the function of other genes. For example, in the *lesser internode number 1* (*lin1*) mutant, the number of rachis internodes is reduced compared to wild-type plants, suggesting that the apical meristem terminates its activity prematurely (Franckowiak and Lundqvist 2002). Defects in spike development with decreased number of rachis internodes and fertile spikelets also characterize the *tip sterile 2* (*tst2*) mutant (Dahleen et al. 2007).

4.3.3.2 Rachis Length and Spike Density

Rachis length and spike density (number of spikelets per length unit) are generally correlated traits; longer rachis correlates positively with longer internodes and hence negatively with spike density. The breeding challenge is to break these constraints, e.g., to generate barley lines with long, dense spikes (and short, sturdy stems to prevent lodging caused by heavy spikes). Rachis development is influenced by allelic variation at a number of loci resulting in a range of phenotypes from lax to compact spikes, often associated with alterations of other plant traits.

The extreme *laxatum* phenotype named *accordion rachis* (Fig. 4.3) is characterized by lax spikes with strongly elongated and pleated rachis internodes and is controlled by three interacting loci, the recessive *accordion rachis1* (*acr1*) on chromosome 2HL, and two incompletely dominant loci, *Acr2* (chromosome 4HL) and *Acr3* (chromosome 1HL). The accordion rachis phenotype can be induced by ectopically expressing two of the barley MADS1 SVP-like (short vegetative phase) genes BM1 and BM10 (Trevaskis et al. 2007). Pleiotropic effects are associated with mutations at the *accordion basal rachis internode 1* (*abr1*) locus. Depending on environmental conditions and genetic background, rachis internodes in the upper part of the spike are longer than normal, and secondary and tertiary shoots may develop from the accordion-like basal rachis nodes (Lundqvist et al. 1996). Lax spikes are also typical of the recessive *laxatum* mutants that exhibit additional defects in plant height and spikelet number (*lax-b*), grain morphology, and tiller number (*lax-c*; Lundqvist et al. 1996). Recessive alleles at *long basal rachis internode* loci (*lbi1*, *lbi2*, *lbi3*) cause pronounced elongation of the first rachis internode (Lundqvist et al. 1996; Franckowiak and Lundqvist 2010). In the recessive *opposite spikelets1* (*ops1*) mutant, spike internodes vary in length resulting in an irregular arrangement of spikelets along the rachis (Franckowiak and Lundqvist 2002). The *ops1* mutation also reduces tiller number.

Compact spikes due to shorter rachis internodes are associated with recessive *erectoides* (*ert*), *dense spike* (*dsp*), *brachytic* (*brh*), *uzu* (*semibrachytic*), *pyramidatum1* (*pyr1*), and (semi) dominant *Zeocriton* (*Zeo*) loci (Fig. 4.3; Forster et al. 2007; Shahinnia et al. 2012). Some of these mutants show general defects in internode elongation that, in some cases, can be rescued by gibberellin treatments indicating they may be defective in gibberellin metabolism (e.g., *brh1*). Pleiotropic effects on plant stature, coleoptile, leaf, awn, glume, and seed length are also typical for the *uzu* gene, encoding HvBRI1, a putative brassinosteroid receptor (Chono et al. 2003), reinforcing the link between spike density and hormonal signaling. Despite the abundance of mutants affecting this trait, *uzu* is the only barley gene involved in control of rachis internode length that has been isolated. The *dsp.ar* mutation was recently fine-mapped to a 0.37 cM interval in the centromeric region of chromosome 7H and may represent an allele of the *dps1* locus (Shahinnia et al. 2012; Taketa et al. 2011).

4.3.3.3 Specification of Spikelet Meristem Identity and Spike Branching

Identities and determinacy of inflorescence, spikelet, and/or floral organ meristems are affected in the mutant group known as *branched* or *compositum*. These phenotypes are controlled by a number of genes and alleles that similar to vegetative branching (tillering) mutants exhibit a wide range of phenotypic variation, with extreme alleles resulting in almost sterile spikes (Fig. 4.3).

Recessive mutations at the *branched1* (*brc1*, chromosome 2HS) and *compositum* (*com1*, chromosome 5HL, *com2*, chromosome 2HS) loci cause the development of

branches from rachis nodes in the basal portion of the spike (Franckowiak and Lundqvist 2010; Druka et al. 2011). Linkage mapping and comparative genomics analyses with rice identified *FRIZZY PANICLE* (*FZP*) as a likely candidate gene for the *brc1* locus on chromosome 2HS; *com2* is believed to be allelic to *brc1* (Rossini et al. 2006, and unpublished results). Although in principle spike branching mutants could be exploited to increase grain number, floret sterility and low seed set is often observed in *brc1* plants (Rossini et al. unpublished). Recently, manipulation of grain number and increased productivity was achieved by silencing of the *HvCKX1* gene, encoding a cytokinin (CK) oxidase/dehydrogenase involved in hormone catabolism (Zalewski et al. 2010). However, it is not clear if this increased productivity (number of seeds per plant and 1,000 kernels weight) is mediated by elevated CK levels as seen in a similar study in rice (Ashikari et al. 2005).

In the *multiflorus 2* (*mul2*) mutant, lateral spikelets may develop extra florets, and occasionally branches form at random in the spike, indicating a possible role for this gene in correct spikelet meristem function (Lundqvist et al. 1996). Modification of spike architecture is also seen in the *rattail spike1* (*rtt1*) mutant, displaying numerous immature spikelets, complete sterility, and possibly the presence of numerous florets within each spikelet (Lundqvist et al. 1996).

4.3.3.4 Specification of Lateral Floret Fate and Row Type

A major partition in barley germplasm is between two- and six-rowed cultivars, partly reflecting breeding history (Cockram et al. 2010; Pasam et al. 2012). For malting and brewing, two-rowed cultivars have traditionally been preferred in Europe (Garstang and Spink 2011), while distinct groups of two- and six-rowed cultivars have been used in the USA (Horsley and Harvey 2011). On the other hand, grains from six-rowed barleys are associated with reduced *in rumen* fermentation rate and therefore have improved livestock feed quality (Blake et al. 2011), and six-rowed barley is often grown as animal feed, for example, in Africa (Palmer et al. 2009). Although six-rowed spikes could potentially produce three times more seeds than two-rowed ones, grain yield is comparable in both mostly due to compensating effects on tiller number (von Bothmer and Komatsuda 2011; Druka unpublished).

At least ten genes with multiple alleles have been identified in mutation screens as controlling row type in barley (Lundqvist and Lundqvist 1988). Some of them are shown in Fig. 4.3, highlighting that different row type genes affect different degrees of lateral floret development of the spike. Probably, the most extreme and interesting case is represented by the *vrs1.t* allele, which, in contrast to the rest of the other known recessive *vrs1* alleles that promote lateral floret development, severely suppresses lateral floret development resulting in a phenotype called *deficiens* (Fig. 4.3).

The two-rowed spike is the ancestral state present in wild barley (*Hordeum vulgare* ssp. *spontaneum*), where it is an adaptive trait facilitating seed dispersal and burial (Sakuma et al. 2011). Six-rowed types arose in cultivated barley by

loss-of-function mutations in *Vrs1*, a gene on chromosome 2HL encoding class I homeodomain-leucine zipper (HD-Zip) transcription factor HvHOX1 required to suppress lateral floret development (Komatsuda et al. 2007). Consistent with this, *Vrs1* is expressed in the two lateral floral meristems in the developing two-rowed spike from the triple-mound stage. Comparison of *Vrs1* gene sequences from wild and cultivated barley accessions indicated that the six-rowed trait arose on at least four independent occasions (Komatsuda et al. 2007; Saisho et al. 2009). The paralogous gene *HvHox2* has been isolated, and it exhibits distinct expression patterns to *Vrs1* (Sakuma et al. 2007). Two possible models of interaction between the HvHOX1 (VRS1) and HvHOX2 proteins have been proposed. One model is that there is differential regulation of downstream targets in the central and lateral spikelet meristems, and an alternative model is that the HvHOX1 protein outcompetes HvHOX2 (Sakuma et al. 2011).

Other recessive loci known to modify the extent of lateral floret development include *Vrs2*, *Vrs3*, and *Vrs4*, located on chromosome 5HL, 1HL, and 3HL, respectively (reviewed in Pourkheirandish and Komatsuda 2007). Both Mendelian and quantitative phenotypic variation are associated with these loci (Franckowiak and Lundqvist 2010; Ramsay et al. 2011; Pasam et al. 2012), but isolation of the underlying genes has not been reported yet.

In two-rowed barley, lateral spikelet development is affected by another set of loci represented by the *intermedium spike* (*int*) mutants (Franckowiak and Lundqvist 2010). Among them, the *Int-c* gene on chromosome 4HS has been associated with natural quantitative variation for row type (Ramsay et al. 2011). Different combinations of *Vrs1* and *Int-c* alleles are found in barley germplasm leading to various levels of lateral spikelet development and fertility (Ramsay et al. 2011). Thus, the recessive *int-c.b* allele is commonly found in two-rowed (*Vrs1*) cultivars where it inhibits anther development in lateral florets, while in six-rowed (*vrs1*) cultivars it results in reduced lateral spikelet development. The dominant *Int-c.a* allele is present in six-rowed (*vrs1*) cultivars, but in two-rowed cultivars, *Int-c.a* produces partially fertile lateral florets, typical of the *intermedium spike* phenotype. The *Int-c* gene was shown to encode the barley ortholog of the maize *TEOSINTE BRANCHED1* (*TB1*) gene, a major regulator of plant architecture changes that accompanied maize domestication (Ramsay et al. 2011). Similar to its maize and rice counterparts (Doebley et al. 1997; Takeda et al. 2003), *Int-c* appears to act in the control of lateral meristem functions with effects also on tillering (Ramsay et al. 2011).

A recent study explored allelic diversity of the *Vrs1* and *Int-c* genes in a large set of *labile* barleys from Ethiopia, characterized by row-type variation within individual spikes of the same genotypes (Youssef et al. 2012). All these accessions possessed a six-rowed genetic background, suggesting that reduced lateral spikelet fertility derives from recessive alleles of a novel locus.

4.3.3.5 Differentiation of Spikelet Organs: Lemma and Awn

Barley genetic collections offer a wide range of mutants altered in the morphology of spikelets and floral organs, especially glumes and bracts (Franckowiak and Lundqvist 2010; Druka et al. 2011). Awns play an important role in seed dispersal in wild cereals (Elbaum et al. 2007) and have long been considered to contribute significantly to grain filling and yield (Taketa et al. 2011 and references therein). On the other hand, in the case of barley cultivars used for feed, the presence of awns is undesired as it can cause jaw infections in animals (Blake et al. 2011). While cultivated barley is mostly full awned, short-awned types are frequently observed in eastern Asia (Takahashi 1987). One of the major genes promoting awn development is *Awnless1* (*Lks1*). The recessive *lks1.a* allele inhibits awn development not only on the lemma (Fig. 4.3) but also on the palea and outer glumes, although *lks1* has no effect on third outer glume awns (Druka unpublished). Awn length is reduced by *short awn* (*lks2*, *lks5*) as well as *breviaristatum* (*ari*) alleles, often with pleiotropic effects on other plant organs (Lundqvist et al. 1996; Druka et al. 2011). Other loci affecting lemma-awn development include dominant *Hooded* (*Kap1*, widespread in forage barley, Blake et al. 2011), recessive *calcaroides* (*cal-b*, *cal-c*, *cal-d*, *cal-e*), *subjacent hood 1* (*sbk1*, previously *cal-a*), *leafy lemma1* (*lel1*), and *triple awned lemma* (*trp1*; Pozzi et al. 2000; Druka et al. 2011; Franckowiak and Lundqvist 2010; Fig. 4.3). Among these, the first to be characterized at the molecular level was *Hooded*, which was associated with a dominant mutation in the barley KNotted-like homeobOX (KNOX) gene *Bkn3* on chromosome 4HS (Müller et al. 1995). A duplication of 305 bp in intron IV of the gene was shown to cause mis-expression of *Bkn3* at the distal end of the lemma, leading to the development of an ectopic floral meristem and consequently an epiphyllous floret ("hood") in place of the awn. The 305-bp intronic element was proposed to act as an enhancer regulating developmental expression of the gene and mediating the cross talk between the *Bkn3* and ethylene pathways via interactions with specific transcription factors (Santi et al. 2003; Osnato et al. 2010). Five recessive *suppressors of Kap1* (*suK*) loci were identified as capable of restoring normal awn development in the *Kap1* background (Roig et al. 2004). Additional players in the genetic network controlling awn development are represented by the *lks2* locus, known to be epistatic to *Kap1* (http://wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class=image;query=;name=Barley,+kap%2FLks2+phenotype), and the *cal* and *sbk1* mutants, which exhibit characteristic outgrowths at the lemma-awn boundary, partly similar to the "hood" but lacking reproductive organs (Pozzi et al. 2000). Natural recessive variants of the *lks2* gene reduce awn length by 50 % and are widespread in Eastern Asia, possibly offering adaptation to high-precipitation conditions. *Lks2* was recently shown to encode an SHI family transcription factor required for correct pistil development and awn elongation through regulation of cell numbers (You et al. 2012). Thus, suppression of the *Kap* phenotype by *lks2* may be due to reduced cell division at the tip of the lemma, where the "hood" meristem forms.

Extra awns form in the *triple awned lemma1* (*trp1*) mutant and on the palea in plants carrying the *awned palea 1* (*adp1*) allele, where the presence of two awns reflects the two midribs characteristic of this organ (Lundqvist et al. 1996; Dahleen et al. 2007; Fig. 4.3).

4.3.3.6 Differentiation of Spikelet Organs: Glumes

Besides genes involved in lemma-awn development, other loci affect formation and morphology of the palea and glumes (Franckowiak and Lundqvist 2010). Recessive alleles of the *Third outer glume1* (*Trd1*) gene on chromosome 1HL cause the formation of an extra bract subtending the central spikelets in the basal region of the spike (Fig. 4.3; Houston et al. 2012). The *Trd1* gene encodes a GATA-zinc finger transcription factor required for bract suppression, and orthologous genes *Nl1* and *Tsh1* play the same role in rice and maize, respectively, indicating the existence of a conserved mechanism for control of bract development in the grass family (Whipple et al. 2010). Additional genes involved in this process may be identified from other mutants (Houston et al. 2012). For example, *bracteatum* (*bra-a*, *bra-d*) and *extra floret* (*flo-a*, *flo-b*, *flo-c*) also develop supernumerary bracts at the base of the central spikelet, occasionally leading to the formation of extra florets (Lundqvist et al. 1996).

4.3.3.7 Differentiation of Floral Organs: Reproductive Organs and Lodicules

Differentiation of lodicules, stamens, and ovary is under genetic control as demonstrated by mutants exhibiting defects or alterations in the development of these structures. For example, in the *lax-a* mutant the two lodicules are transformed into two small anthers containing two microsporangia compared to the four present in the proper anthers (Bossinger et al. 1992a). Lodicule development is also under the control of the *Cly1* gene (Nair et al. 2010). *Cly1* encodes an AP2 transcription factor likely functioning as a repressor of lodicule development. In chasmogamous barley plants, cleavage of the *Cly1* mRNA, guided by miR172, results in downregulation of *Cly1* gene expression and growth and swelling of the lodicules leading to exposed reproductive organs. Recessive *cly1* alleles restrict lodicule development and cause cleistogamy, i.e., the tight association of the palea and lemma that prevents anther exertion and cross-pollination. In cleistogamous lines, a mutation at the miRNA-binding site prevents mRNA cleavage, leading to increased *Cly1* expression and reduced lodicules.

A number of interesting floral organ development mutants have been identified during mutant screens by careful examination of the floral structures (http://barleygenomics.wsu.edu/mut-4-3-2.html). Those include extreme mutations that lead to complete loss of floral reproductive organs (*no reproductive organs*, *ovaryless*, *bushy spike1*), altered morphology (*unbranched style*), or floral organ

conversions (*lodiculeless*, which has been originally identified as one of the *laxatum* mutants) (Bossinger et al. 1992a).

Stamens are converted into carpels in recessive *multiovary* (*mov*) mutants, leading to the formation of supernumerary carpels within the floret. In the case of *mov1*, lodicules are also transformed into leaflike structures suggesting that this gene plays a role in specification of two adjacent whorls (Franckowiak et al. 2005). Conversely, lodicules appear normal in *mov2*, *mov3* and *mov4*. Five to seven carpels can be observed in *mov2* plants, while *mov4* lines produce two anthers and two carpels, sometimes giving rise to two seeds from one floret (Franckowiak et al. 2005).

The development of the ovary is controlled by *ovaryless* (*ovl*) genes (Franckowiak et al. 2005). Ovaries are absent in the *ovl1* and *ovl2* mutants, while anthers are normal and pollen is viable (Fig. 4.3). Additional defects of *ovl1* plants include lack of awns and midribs in leaves, indicating that the *Ovl1* gene may play a more general developmental role.

4.4 Exploiting Genes Regulating Shoot and Inflorescence Architecture for Crop Improvement

The understanding of shoot and inflorescence development and the genetic control of these processes have the potential to increase the efficiency of barley improvement programs. As described above, numerous genes controlling development have been isolated, and more will be identified soon. Genetic manipulation of these developmental processes could positively impact grain yield, vegetative biomass, and lodging resistance. In the future, barley improvement based on an understanding of the genes controlling the developmental phenotype will be conducted following two basic strategies, namely, (1) identifying beneficial alleles in germplasm collections and utilizing the alleles in breeding programs and (2) developing transgenic plants with these genes. In this section, we briefly describe previous efforts to conduct ideotype breeding for increased grain yield and propose how ideotype breeding could be informed by understanding the genetic regulation of plant development. Then, we discuss two modern approaches to gene-based crop improvement.

Ideotype breeding seeks to define traits that will lead to increased yield, define the goals for those traits, and select directly on those traits (Rasmusson 1987). The theoretical framework of ideotype breeding was developed in the 1960s (Donald 1968). A model plant is defined, and the breeder attempts to select for that model plant. Ideotype breeding was first proposed by Donald (1968) to increase wheat yield in a high-input environment. Donald proposed that wheat breeders select for a single culm, short stem, few erect leaves and spike, awns, and high harvest index. There are three major reasons for conducting ideotype breeding including: (1) the increased yield associated with semidwarf wheat and rice (Khush 2001), (2) single

traits directly or indirectly can increase yield, and (3) it can bridge the divide between unimproved germplasm and advanced breeding germplasm (Rasmusson 1987). Donald (1979) and Rasmusson (1987, 1991) pioneered and championed ideotype breeding in barley with mixed results. Several factors impeded the breeding progress using an ideotype approach including (1) symmetry between selected structures, (2) compensation among structures, (3) pleiotropy, and (4) genetic background (Rasmusson 1987). An understanding of the genetic regulation of development may help breeders overcome some of these difficulties.

The development of genomics tools will provide the foundation for isolating and integrating beneficial alleles into barley breeding programs and retesting the ideotype breeding approach. The barley genome sequence (International Barley Genome Sequencing Consortium 2012), next-generation sequencing technologies, and other associated genomic-enabling tools provide the ability to quickly isolate genes underlying mutant phenotypes and mine germplasm collections for beneficial alleles. The advent of these genomics tools coupled with our increased understanding of the genetic control of development will provide the opportunity to select on single genes that control single traits. Thus, in the future it may be possible to combine ideotype breeding with the appropriate selection of desirable alleles that control developmental phenotypes, resulting in increased productivity.

Genetic engineering provides the opportunity to assess genes from other species and thus allows the ability to incorporate genetic variation outside that contained in barley germplasm collections. Barley transformation is routine in several laboratories around the world (e.g., Dahleen and Manoharan 2007). For details on barley transformation, see Chap. 21 of this book. The current status of barley transformation provides the opportunity to over-, under-, and mis-express transgenes that alter shoot and inflorescence architecture. Instructive examples include the expression of the maize *Tb1* and rice *OsTB1* genes in wheat and rice that resulted in reduced tillering, respectively (Lewis et al. 2008; Takeda et al. 2003), indicating that it is possible to dramatically alter plant morphology. Another example includes silencing of the *HvCKX1* gene resulting in increased grain number and productivity (Zalewski et al. 2010). These examples demonstrate just the beginning of the ability to tap the tremendous potential for altering plant development with the goal to increase yield and biomass.

References

Arite T, Ohshima IH, Maekawa K, Nakajima M, Kojima M, Sakakibara H, Kyozuka J (2007) *Dwarf10*, an *RMS1/MAX4/DAD1* ortholog, controls lateral bud outgrowth in rice. Plant J 51: 1019–1029

Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q, Kitano H, Matsuoka M (2005) Cytokinin oxidase regulates rice grain production. Science 309: 741–745

Babb S, Muehlbauer GJ (2003) Genetic and morphological characterization of the barley *uniculm2* (*cul2*) mutant. Theor Appl Genet 106:846–857

Barnard C (1964) Form and structure. In: Barnard C (ed) Grasses and grasslands. MacMillan, London, pp 47–72

Baum M, Grando S, Backes G, Jahoor A, Sabbagh A, Ceccarelli S (2003) QTLs for agronomic traits in the Mediterranean environment identified in recombinant inbred lines of the cross 'Arta' x *H. spontaneum* 41-1. Theor Appl Genet 107:1215–1225

Blake T, Blake VC, Bowman JGP, Abdel-Haleem H (2011) Barley feed uses and quality improvement. In: Ullrich SE (ed) Barley: production, improvement, and uses. John Wiley-Blackwell, West Sussex, pp 522–531

Borem A, Mather DE, Rasmusson DC, Fulcher RG, Hayes PM (1999) Mapping quantitative trait loci for starch granule traits in barley. J Cereal Sci 29:153–160

Bossinger G, Rohde W, Lundqvist U, Salamini F (1992a) Genetics of barley development: mutant phenotypes and molecular aspects. In: Shewry PR (ed) Barley: genetics, biochemistry, molecular biology and biotechnology. CAB International, Wallingford, pp 231–263

Bossinger G, Lundqvist U, Rohde W, Salamini F (1992b) Genetics of plant development in barley. In: Munck L (ed) Barley genetics VI, vol II. Munksgaard International, Copenhagen, pp 989–1017

Bowman JL, Eshed Y (2000) Formation and maintenance of the shoot apical meristem. Trends Plant Sci 5:110–115

Brand U, Hobe M, Simon R (2001) Functional domains in plant shoot meristems. Bioessays 23:134–141

Byrne ME, Groover AT, Fontana JR, Martienssen RA (2003) Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene *BELLRINGER*. Development 130:3941–3950

Chandler PM, Harding CA (2013) 'Overgrowth' mutants in barley and wheat: new alleles and phenotypes of the 'Green Revolution' Della gene. J Exp Bot 64(6):1603–1613. doi:10.1093/jxb/ert022

Chandler PM, Robertson M (1999) Gibberellin dose-response curves and the characterization of dwarf mutants of barley. Plant Physiol 120:623–632

Chandler PM, Marion-Poll A, Ellis M, Gubler F (2002) Mutants at the *Slender1* locus of barley cv. Himalaya. Molecular and physiological characterization. Plant Physiol 129:181–190

Chono M, Honda I, Zeniya H, Yoneyama K, Saisho D, Takeda K, Takatsuto S, Hoshino T, Watanabe Y (2003) A semidwarf phenotype of barley *uzu* results from a nucleotide substitution in the gene encoding a putative brassinosteroid receptor. Plant Physiol 133:1209–1219

Clark LG, Fisher JB (1988) Vegetative morphology of grasses. In: Soderstrom TR, Hiu KW, Campbell CS, Barkworth ME (eds) Grass systematics and evolution. Smithsonian Institution Press, Washington, DC, pp 37–45

Clifford HT (1988) Spikelet and floral morphology. In: Soderstrom TR, Hiu KW, Campbell CS, Barkworth ME (eds) Grass systematics and evolution. Smithsonian Institution Press, Washington, DC, pp 21–30

Close TJ, Wanamaker S, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP (2004) A new resource for cereal genomics: 22K barley GeneChip comes of age. Plant Physiol 134:960–968

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou M, Chao S, Varshney RK, Szücs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582

Cockram J, White J, Zuluaga DL, Smith D, Comadran J, Macaulay M, Luo Z, Kearsey MJ, Werner P, Harrap D, Tapsell C, Liu H, Hedley PE, Stein N, Schulte D, Steuernagel B, Marshall DF, Thomas WT, Ramsay L, Mackay I, Balding DJ, AGOUEB Consortium, Waugh R, O'Sullivan DM (2010) Genome-wide association mapping to candidate polymorphism resolution in the unsequenced barley genome. Proc Natl Acad Sci U S A 107:21611–21616

Cole M, Nolte C, Werr W (2006) Nuclear import of the transcription factor *SHOOT MERISTEMLESS* depends on heterodimerization with BLH proteins expressed in discrete sub-domains of the shoot apical meristem of Arabidopsis thaliana. Nucleic Acids Res 34: 1281–1292

Dabbert T, Okagaki RJ, Cho S, Boddu J, Muehlbauer GJ (2009) The genetics of barley low-tillering mutants: *absent lower laterals* (*als*). Theor Appl Genet 118:1351–1360

Dabbert T, Okagaki RJ, Cho S, Heinen S, Boddu J, Muehlbauer GJ (2010) The genetics of barley low-tillering mutants: *low number of tillers-1* (*lnt1*). Theor Appl Genet 121:705–717

Dahleen LS, Manoharan M (2007) Recent advances in barley transformation. InVitro Cell Dev Plant 43:493–506

Dahleen L, Franckowiak JD, Lindqvist U (2007) Barley genetic stocks for 2007. Barley Genet Newsl 37:188–301

Dahlgren RHT, Clifford HT, Yeo PF (1985) The families of the monocotyledons. Structure, evolution and taxonomy. Springer, New York, NY, 520 pp

de la Peña R, Smith KP, Capettini F, Muehlbauer GJ, Gallo-Meagher M, Dill-Macky R, Somers DA, Rasmusson DC (1999) Quantitative trait loci associated with resistance to *Fusarium* head blight and kernel discoloration in barley. Theor Appl Genet 99:561–569

Doebley J, Stec A, Hubbard L (1997) The evolution of apical dominance in maize. Nature 386: 485–488

Domagalska MA, Leyser O (2011) Signal integration in the control of shoot branching. Nat Rev Mol Cell Biol 12:211–221

Donald CM (1968) The breeding of crop ideotypes. Euphytica 17:385–403

Donald CM (1979) A barley breeding programme based on ideotype. J Agric Sci Camb 93: 261–269

Döring HP, Lin J, Uhrig H, Salamini F (1999) Clonal analysis of the development of the barley (*Hordeum vulgare* L.) leaf using periclinal chlorophyll chimeras. Planta 207:335–342

Druka A, Druka I, Centeno AG, Li H, Sun Z, Thomas WT, Bonar N, Steffenson BJ, Ullrich SE, Kleinhofs A, Wise RP, Close TJ, Potokina E, Luo Z, Wagner C, Schweizer GF, Marshall DF, Kearsey MJ, Williams RW, Waugh R (2008) Towards systems genetic analyses in barley: integration of phenotypic, expression and genotype data into GeneNetwork. BMC Genet 9:73

Druka A, Franckowiak J, Lundqvist U, Bonar N, Alexander J, Houston K, Radovic S, Shahinnia F, Vendramin V, Morgante M, Stein N, Waugh R (2011) Genetic dissection of barley morphology and development. Plant Physiol 155:617–627

Elbaum R, Zaltzman L, Bugert I, Fratzl P (2007) The role of wheat awns in the seed dispersal unit. Science 316:884

Forster BP, Franckowiak JD, Lundqvist U, Lyon J, Pitkethly I, Thomas WTB (2007) The barley phytomer. Ann Bot 100:725–733

Franckowiak JD (1987) Coordinator's report on the semi-dwarf barley collection. Barley Genet Newsl 17:114–115

Franckowiak JD (2000) Notes on plant height in six-rowed barley and FHB resistance. In: Barley genetics VIII: Proceedings of the 8th international barley genetics symposium, vol 2, pp 107–109

Franckowiak JD, Lundqvist U (2002) New and revised barley genetic stock descriptions. Barley Genet Newsl 32:49–137

Franckowiak JD, Lundqvist U (2010) Descriptions of barley genetic stocks for 2010. Barley Genet Newsl 40:45–177

Franckowiak JD, Pecio A (1992) Coordinator's report: a listing of genetic stocks. Barley Genet Newsl 21:116–126

Franckowiak JD, Kleinhofs A, Lundqvist U (2005) Descriptions of barley stocks for 2005. Barley Genet Newsl 35:155–210

Garstang JR, Spink JH (2011) Cultural practices: focus on major barley-producing regions. Europe. In: Ullrich SE (ed) Barley: production, improvement, and uses. John Wiley-Blackwell, West Sussex, pp 221–231

Gubatz S, Shewry PR (2011) The development, structure and composition of the barley grain. In: Ullrich SE (ed) Barley: production, improvement, and uses. John Wiley-Blackwell, West Sussex, pp 391–448

Gyenis L, Yun SJ, Smith KP, Steffenson BJ, Bossolini E, Muehlbauer GJ (2007) Genetic architecture of quantitative trait loci associated with morphological and agronomic trait differences in a wild by cultivated barley cross. Genome 50:714–723

Hayes PM, Liu BH, Knapp SJ, Chen F, Jones B, Blake T, Franckowiak J, Rasmusson D, Sorrells M, Ullrich SE, Wesenberg D, Kleinhofs A (1993) Quantitative trait locus effects and environmental interaction in a sample of North American barley germplasm. Theor Appl Genet 87:392–401

Hellewell KB, Rasmusson DC, Gallo-Meagher M (2000) Enhancing yield of semidwarf barley. Crop Sci 40:352–358

Horsley RD, Harvey BL (2011) Barley breeding history, progress, objectives, and technology. North America. In: Ullrich SE (ed) Barley: production, improvement, and uses. John Wiley-Blackwell, West Sussex, pp 171–186

Hoskins PH, Poehlman JM (1971) Pleiotropic effects of *uzu* and spike-density genes in a barley cross. J Hered 62:153–156

Houston K, Druka A, Bonar N, Macaulay M, Lundqvist U, Franckowiak J, Morgante M, Stein N, Waugh R (2012) Analysis of the barley bract suppression gene Trd1. Theor Appl Genet 125: 33–45

International Barley Genome Sequencing Consortium (2012) A physical, genetic and functional sequence assembly of the barley genome. Nature 491:711–716

Ishikawa S, Maekawa M, Arite T, Onishi K, Takamure I, Kyozuka J (2005) Suppression of tiller bud activity in tillering dwarf mutants of rice. Plant Cell Physiol 46:79–86

Jia Q, Zhang XQ, Wescott S, Broughton S, Cakir M, Yang J, Lance R, Li C (2009) Expression level of a gibberellin 20-oxidase gene is associated with multiple agronomic and quality traits in barley. Theor Appl Genet 122:1451–1460

Khush GS (2001) Green revolution: the way forward. Nat Rev Genet 2:815–822

Kirby EJM, Appleyard M (1987) Cereal Development Guide, 2nd edn. Arable Unit, Stoneleigh, Warwickshire

Kleinhofs A, Kilian A, Saghai Maroof MA, Biyashev RM, Hayes P, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705–712

Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Matsumoto T, Yano M (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci U S A 104:1424–1429

Konishi S, Izawa T, Lin SY, Ebana K, Fukuta Y, Sasaki T, Yano M (2006) An SNP caused loss of seed shattering during rice domestication. Science 312:1392–1396

Lewis JM, Mackintosh CA, Gilding E, Shin S, Kravchenko S, Baldridge G, Zeyen R, Muehlbauer GJ (2008) Overexpression of maize *Teosinte Branched1* in wheat suppresses tiller development. Plant Cell Rep 27:1217–1225

Leyser O, Day S (2003) Mechanisms in plant development. Blackwell, Oxford

Long J, Barton MK (2000) Initiation of axillary and floral meristems in Arabidopsis. Dev Biol 218: 341–353

Lundqvist U, Lundqvist A (1988) Induced *intermedium* mutants in barley: origin, morphology and inheritance. Hereditas 108:13–26

Lundqvist U, Franckowiak J, Konishi T (1996) New and revised descriptions of barley genes. Barley Genet Newsl 26:22

MacLeod AM, Palmer GH (1966) The embryo of barley in relation to modification of the endosperm. J Inst Brew 72:580–589

Marquez-Cedillo LA, Hayes PM, Kleinhofs A, Legge WG, Rossnagel BG, Sato K, Ullrich SE, Wesenberg DM (2001) QTL analysis of agronomic traits in barley based on the doubled haploid progeny of two elite North American varieties representing different germplasm groups. Theor Appl Genet 103:625–637

McSteen P, Leyser O (2005) Shoot branching. Annu Rev Plant Biol 56:353–374

Mickelson HR, Rasmusson DC (1994) Genes for short stature in barley. Crop Sci 34:1180–1183

Müller KJ, Romano N, Gerstner O, Garcia-Marotot F, Pozzi C, Salamini F, Rohde W (1995) The barley *Hooded* mutation caused by a duplication in a homeobox gene intron. Nature 374: 727–730

Müller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W (2001) In vitro interactions between barley TALE homeodomain proteins suggest a role for protein-protein associations in the regulation of Knox gene function. Plant J 27:13–23

Nair SK, Wang N, Turuspekov Y, Pourkheirandish M, Sinsuwongwat S, Chen G, Sameri M, Tagiri A, Honda I, Watanabe Y, Kanamori H, Wicker T, Stein N, Nagamura Y, Matsumoto T, Komatsuda T (2010) Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. Proc Natl Acad Sci U S A 107:490–495

Okagaki RJ, Cho S, Kruger WM, Xu WW, Heinen S, Muehlbauer GJ (2013) Structural and transcriptional analysis of the barley *uniculm2* mutant. Funct Integr Genomics 13:33–41

Osnato M, Stile MR, Wang Y, Meynard D, Curiale S, Guiderdoni E, Liu Y, Horner DS, Ouwerkerk PB, Pozzi C, Müller KJ, Salamini F, Rossini L (2010) Cross talk between the KNOX and ethylene pathways is mediated by intron-binding transcription factors in barley. Plant Physiol 154:1616–1632

Palmer SA, Moore JD, Clapham AJ, Rose P, Allaby RG (2009) Archaeogenetic evidence of ancient nubian barley evolution from six to two-row indicates local adaptation. PLoS One 4(7): e6301

Pasam RK, Sharma R, Malosetti M, van Eeuwijk FA, Haseneyer G, Kilian B, Graner A (2012) Genome-wide association studies for agronomical traits in a world wide spring barley collection. BMC Plant Biol 12:16

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD, Harberd NP (1999) "Green revolution" gene encode mutant gibberillin response modulators. Nature 400:256–261

Pourkheirandish M, Komatsuda T (2007) The importance of barley genetics and domestication in a global perspective. Ann Bot 100:999–1008

Pozzi C, Faccioli P, Terzi V, Stanca AM, Cerioli S, Castiglioni P, Fink R, Capone R, Müller KJ, Bossinger G, Rohde W, Salamini F (2000) Genetics of mutations affecting the development of a barley floral bract. Genetics 154:1335–1346

Pozzi C, di Pietro D, Halas G, Roig C, Salamini F (2003) Integration of a barley (*Hordeum vulgare*) molecular linkage map with the position of genetic loci hosting 29 developmental mutants. Heredity 90:390–396

Qi X, Nicks EE, Stam P, Lindhourt P (1998) Identification of QTLs for partial resistance to leaf rust (*Puccinia hordei*) in barley. Theor Appl Genet 96:1205–1213

Ramsay L, Comadran J, Druka A, Marshall DF, Thomas WTB, Macaulay M, MacKenzie K, Simpson C, Fuller J, Bonar N, Hayes PM, Lundqvist U, Franckowiak JD, Close TJ, Muehlbauer GJ, Waugh R (2011) *INTERMEDIUM-C* a modifier of lateral spikelet fertility in barley, is an ortholog of the maize domestication gene *TEOSINTE BRANCHED 1*. Nat Genet 43:169–172

Rasmusson DC (1987) An evaluation of ideotype breeding. Crop Sci 27:1140–1146

Rasmusson DC (1991) A plant breeder's experience with ideotype breeding. Field Crops Res 26: 191–200

Roeder AHK, Ferrándiz C, Yanofsky MF (2003) The role of the REPLUMLESS homeodomain protein in patterning the Arabidopsis fruit. Curr Biol 13:1630–1635

Roig C, Pozzi C, Santi L, Müller J, Wang Y, Stile MR, Rossini L, Stanca M, Salamini F (2004) Genetics of barley *Hooded* suppression. Genetics 167:439–448

Rossini L, Vecchietti A, Nicoloso L, Stein N, Franzago S, Salamini F, Pozzi C (2006) Candidate genes for barley mutants involved in plant architecture: an *in silico* approach. Theor Appl Genet 112:1073–1085

Rudall PJ, Stuppy W, Cunniff J, Kellogg EA, Briggs BG (2005) Evolution of reproductive structures in grasses (Poaceae) inferred by sister-group comparison with their putative closest living relatives, Ecdeiocoleaceae. Am J Bot 9:1432–1443

Rutjens B, Bao D, van Eck-Stouten E, Brand M, Smeekens S, Proveniers M (2009) Shoot apical meristem function in Arabidopsis requires the combined activities of three Bel1-like homeodomain proteins. Plant J 58:641–654

Saisho D, Tanno K, Chono M, Honda I, Kitano H, Takeda K (2004) Spontaneous brassinolide-insensitive barley mutants '*uzu*' adapted to East Asia. Breed Sci 54:409–416

Saisho D, Pourkheirandish M, Kanamori H, Matsumoto T, Komatsuda T (2009) Allelic variation of row type gene *Vrs1* in barley and implication of the functional divergence. Breed Sci 59: 621–628

Sakuma S, Pourkheirandish M, Matsumoto T, Koba T, Komatsuda T (2007) Duplication of a well-conserved homeodomain-leucine zipper transcription factor gene in barley generates a copy with more specific functions. Funct Integr Genomics 10:123–133

Sakuma S, Salomon B, Komatsuda T (2011) The domestication syndrome genes responsible for the major changes in plant form in the Triticeae crops. Plant Cell Physiol 52:738–749

Santi L, Wang Y, Stile MR, Berendzen K, Wanke D, Roig C, Pozzi C, Müller KJ, Müller J, Rohde W, Salamini F (2003) The GA octodinucleotide repeat binding factor BBR participates to the transcriptional regulation of selected plant homeobox genes. Plant J 34:813–826

Schmitz G, Theres K (2005) Shoot and inflorescence branching. Curr Opin Plant Biol 8:506–511

Sears RG, Kronstad WE, Metzger RJ (1981) Inheritance of dwarf and semidwarf plant height in barley. Crop Sci 21:828–833

Shahinnia F, Druka A, Franckowiak J, Morgante M, Waugh R, Stein N (2012) High resolution mapping of *Dense spike-ar* (*dsp.ar*) to the genetic centromere of barley chromosome 7H. Theor Appl Genet 124:373–384

Sharman BC (1942) Developmental anatomy of the shoot of *Zea mays* L. Ann Bot 6:245–282

Smith HMS, Hake S (2003) The interaction of two homeobox genes, *BREVIPEDICELLUS* and *PENNYWISE*, regulates internode patterning in the Arabidopsis inflorescence. Plant Cell 15: 1717–1727

Sreenivasulu N, Schnurbusch T (2012) A genetic playground for enhancing grain number in cereals. Trends Plant Sci 17:91–101

Swenson SP, Wells DG (1944) The linkage relations of four genes in chromosome I of barley. J Am Soc Agron 36:429–435

Takahashi R (1987) Genetic features of East Asian barleys. In: Yasuda S, Konishi T (eds) Barley genetics V. Maruzen, Okayama, pp 7–20

Takeda T, Suwa Y, Suzuki M, Kitano H, Ueguchi-Tanaka M, Ashikari M, Matsuoka M, Ueguchi C (2003) The *OsTB1* gene negatively regulates lateral branching in rice. Plant J 33:513–520

Taketa S, You T, Sakurai Y, Miyake S, Ichii M (2011) Molecular mapping of the *short awn 2* (*lks2*) and *dense spike* (*dsp1*) genes on barley chromosome 7H. Breed Sci 61:80–85

Thomas WTB, Powell W, Wood W (1984) The chromosomal location of the dwarfing gene in the spring variety Golden Promise. Heredity 53:177–183

Trevaskis B, Tadege M, Hemming MN, Peacock WJ, Dennis ES, Sheldon C (2007) Floral meristem identity in barley. Plant Physiol 143:225–235

Tsuchiya T (1976) Allelism testing in barley. II. Allelic relationships of three *uzu* genes. Crop Sci 16:496–499

von Bothmer R, Komatsuda T (2011) Barley origin and related species. In: Ullrich SE (ed) Barley: production, improvement, and uses. John Wiley-Blackwell, West Sussex, pp 14–62

Weatherwax P (1923) The story of the maize plant. University of Chicago Press, Chicago, IL

Whipple CJ, Hall DH, DeBlasio S, Taguchi-Shiobara F, Schmidt RJ, Jackson DP (2010) A conserved mechanism of bract suppression in the grass family. Plant Cell 22:565–578

Williams RF (1975) The shoot apex and leaf growth. Cambridge University Press, Cambridge
You T, Yamashita Y, Kanamori H, Matsumoto T, Lundqvist U, Sato K, Ichii M, Jobling SA, Taketa S (2012) A SHORT INTERNODES (SHI) family transcription factor gene regulates awn elongation and pistil morphology in barley. J Exp Bot 63:5223–5232
Youssef HM, Koppolu R, Schnurbusch T (2012) Re-sequencing of vrs1 and int-c shows that labile barleys (*Hordeum vulgare* convar. Labile) have a six-rowed genetic background. Genet Res Crop Evol 59:1319–1328
Zalewski W, Galuszka P, Gasparuis S, Orczyk W, Nadolska-Orczyk A (2010) Silencing of the *HvCKX1* gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to higher plant productivity. J Exp Bot 61:1839–1851
Zhang J (1994) Analyses on changes in plant height of varieties and dwarf sources of barley breeding in China. Barley Sci 4:11–13
Zhang J (2000) Inheritance of agronomic traits from the Chinese barley dwarfing gene donors Xiaoshan Lixiahuang and Cangzhou Luodamai. Plant Breed 119:523–524
Zhang J, Zhang W (2003) Tracing sources of dwarfing genes in barley breeding in China. Euphytica 131:285–292
Zou J, Zhang S, Zhang W, Li G, Chen Z, Zhai W, Zhao X, Pan X, Lie Q, Zhu L (2006) The rice *HIGH-TILLERING DWARF1* encoding an ortholog of Arabidopsis *MAX3* is required for negative regulation of the outgrowth of axillary buds. Plant J 48:687–698

Chapter 5
Genetic Control of Reproductive Development

Benedikt Drosse, Chiara Campoli, Aman Mulki, and Maria von Korff

5.1 Introduction

Understanding the genetic and environmental control of reproductive development is important for maximising grain yield in cereal breeding programs. This paper reviews the phenology and genetics of meristem development and flowering time in barley. The effects of meristem development on yield component traits are discussed. Barley flowering time genes, loci and functional genetic diversity are presented, and their roles in domestication and adaptation of barley to different cultivation areas are discussed. Functional interactions of flowering time genes in barley are discussed in the light of information on the flowering time pathways in the model plants *Arabidopsis* and rice. Diagnostic markers for functional allelic variants of flowering time genes and QTLs are presented. Finally, we discuss how natural and induced genetic variation can be exploited to maintain continued varietal improvement for current and future agro-environments. A better understanding of the physiological and genetic basis of flowering time, including possible signalling in response to different environmental cues, will be crucial to design future crops.

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5.2 Phenology of Reproductive Development in Barley

Barley is a facultative long-day plant and flowering time is controlled in response to environmental cues, primarily by photoperiod, ambient temperature and vernalisation. In some barley genotypes, short-day accelerates flowering time in a similar way as cold treatment and was therefore referred to as short-day vernalisation (Roberts et al. 1988).

The concept of photothermal time has been introduced to characterise the effects of genetic components and environmental cues, in particular photoperiod and temperature, on cereal plant development (Roberts et al. 1988; Ellis et al. 1988, 1989). To calculate photothermal time, a genotype-dependent, linear relationship of the reciprocal of flowering time (rate of development) to the photoperiod and mean diurnal temperature was established (Roberts et al. 1988; Ellis et al. 1988, 1989). This concept of photothermal time applies only between the base and maximal temperatures as well as between the ceiling and critical photoperiods. Depending on the genotype, the ceiling and the critical photoperiods for barley as a long-day crop were found to be $\leq$10 h day^{-1} and $\geq$13 h day^{-1}, respectively (Roberts et al. 1988). Interaction effects between photoperiod and temperature were also observed, as the critical photoperiod varies with temperature (Ellis et al. 1988; González et al. 2002). Within the photoperiod and temperature constraints, crop modellers have developed ecophysiological quantitative equations for describing the photothermal responses of phenology, in order to predict flowering times of crop genotypes under a range of environmental conditions or to provide the temporal framework for modelling a number of processes in a general crop growth model (Yin 2005).

Unlike ambient temperature, photoperiod and vernalisation have a predominant impact on developmental rate only during certain parts of the pre-flowering period (Slafer and Rawson 1994; González et al. 2002; Whitechurch et al. 2007). Photothermal time thus allows predicting time from germination to flowering; however, different phenological phases of pre-anthesis development differ in their sensitivity to distinct environmental cues.

Pre-anthesis development in temperate cereals has been divided into three phases based on morphological changes of the shoot apical meristem: the vegetative phase, the early reproductive phase and the late reproductive phase (Fig. 5.1, Slafer and Rawson 1994; González et al. 2002), previously described as pre-inductive, inductive and post-inductive phases by Ellis et al. (1988). During the vegetative phase, the crop initiates leaves until floral initiation, which is generally estimated as the formation of the first spikelet primordia, visible as double ridges at the shoot apex of the main shoot. In the subsequent early reproductive phase, the spikelets are differentiated until the initiation of the terminal spikelet in wheat. Finally, during the late reproductive phase, when the stem internodes elongate, the floret primordia reach their maximum number and then mature. During this process, some florets degenerate while others reach the fertile stage at

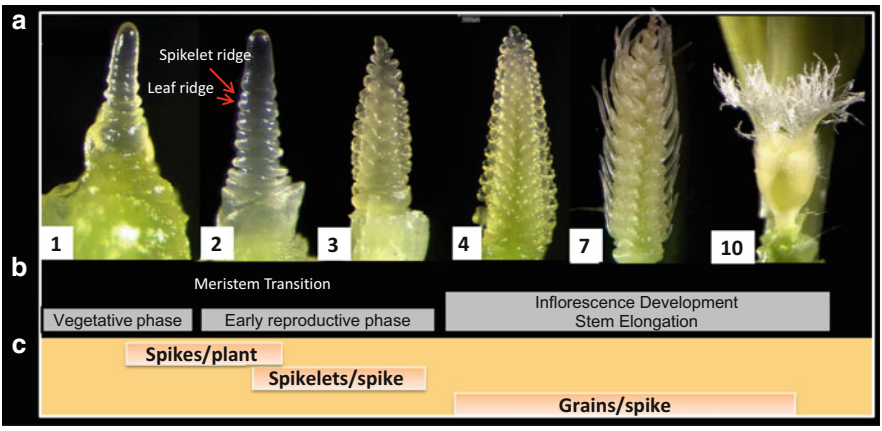

Fig. 5.1 Development of the shoot apical meristem in barley. (**a**) Waddington scale, (**b**) major phases of meristem development, (**c**) yield component traits affected by the duration of meristem phases

anthesis. A quantitative scale for barley development based on the morphogenesis of the shoot apex and florets is provided by Waddington et al. (1983).

Vernalisation affects flowering time, predominantly by reducing the duration of the vegetative phase (Griffiths et al. 1985; Roberts et al. 1988), although minor effects of vernalisation were also reported on the subsequent phases (González et al. 2002). In contrast, long photoperiods had minor effects on the duration of the vegetative phase but strongly accelerated the late reproductive phase of inflorescence development (Roberts et al. 1988; Miralles and Richards 2000). Analyses of wheat development under artificially manipulated photoperiods have shown that the stem elongation phase was the most sensitive to changes in photoperiod (Slafer et al. 2001). Thus, the timing and duration of the different developmental phases vary independently and are determined genetically in response to the environment (González et al. 2003; Whitechurch et al. 2007).

Flowering time integrates the durations of pre-anthesis phases and depends on a timely coordination of morphological changes at the shoot apex, formation of the spike and plant growth, e.g. stem elongation. During the maximum stem and spike growth phase, floret primordia are aborted, which has been attributed to the competition between the spike and stem for limited assimilates (Fig. 5.1, González et al. 2003; Ghiglione et al. 2008; González et al. 2011). Consequently, the duration of stem elongation has been associated with the number of fertile florets (Miralles and Richards 2000; González et al. 2003; Slafer 2003), which is correlated to the spike dry weight at anthesis and sets the final number of grains, the most important component of cereal yield (Reynolds et al. 2009). Slafer et al. (2001) hypothesised that increasing the duration of stem elongation phase would result in a higher number of fertile florets as an alternative to improving wheat yield potential. Increasing the final number of grains may thus be achieved by manipulating the length of different developmental phases so that the same time to flowering may be

achieved, but with a different partitioning of the time between phases occurring before and after the onset of stem elongation.

A better understanding of the physiological and genetic basis of flowering time, including possible signalling in response to different environmental cues, such as photoperiod and temperature, may permit floret abortion to be minimised for a more optimal source-sink balance.

5.3 Variation in Flowering Time and Adaptation

Genetic variation in the vernalisation and photoperiod pathways was crucial for the successful expansion of barley cultivation from the Fertile Crescent to temperate climates. Vernalisation requirement and response are characterised by the temporal separation between the plant's exposure to cold in winter and the onset of flowering in spring and a renewed vernalisation requirement for flowering in subsequent generations. This vernalisation requirement prevents flowering during winter for the protection of the floral organs from cold. After exposure to cold and completed vernalisation, photoperiod sensitivity induces flowering in response to increasing day length.

Barley is characterised by two major growth types: winter and spring. Winter growth types are defined here as genotypes which show accelerated flowering after vernalisation, a prolonged exposure to cold temperature. In contrast, spring barley does not respond to vernalisation. However, there exists a continuous gradation regarding spring and winter growth habits from typical spring to extreme winter (vernalisation requirement) (Rollins et al. 2013). Wild barley *H. vulgare* ssp. *spontaneum*, the progenitor of cultivated barley, originated in the Fertile Crescent and is still a widespread species found over the Eastern Mediterranean basin and Western Asiatic countries. Wild barley is classified as having a winter growth habit and early flowering under long days, indicating that the winter growth habit is ancestral in barley (Saisho et al. 2011). In Mediterranean areas and the Near East, cultivated barley is generally sown in autumn and typically displays a winter growth habit and responds to vernalisation but may also flower eventually in the absence of vernalisation. However, there exists a large variation in growth habits between and within landrace populations from the Fertile Crescent (Weltzien 1988, 1989). The distribution of winter- and spring-type genotypes in the Fertile Crescent coincides with the increasingly continental weather patterns from west and south to east and depends on the use of barley for sheep grazing in some areas. The spring growth type is thus more common in the coastal areas and southern parts of the Fertile Crescent where winter temperatures are mild, but cultivars with and without vernalisation response occupy similar cultivation areas (Yasuda et al. 1993; Saisho et al. 2011). Winter growth types have been selected and improved for cold resistance for cultivation in northern latitudes (Cockram et al. 2007). Spring growth types have been selected and bred for sowing in spring and a reduced photoperiod response for late flowering in summer. Late flowering in temperate environments

with a long growing season allows cereal crops to exploit an extended vegetative period for resource storage. A further expansion of barley cultivation to Northern areas with cold winters and short summers required the selection of early flowering in spring-grown barley. This led to the selection of early flowering genotypes which do not respond to photoperiod or vernalisation and are characterised by the presence of the so-called *earliness per se* (*eps*) or *early maturity* (*eam*) genes. Scandinavian breeding programs used different mutagenic treatments to generate early maturing barley mutants in spring barley backgrounds which produce a day-neutral phenotype with rapid flowering under short- or long-day conditions (Lundqvist 2009). For example, the *eam8* mutation on 1HL generated by mutagenic treatment and detected in natural lines was successfully introduced into breeding lines and released as cultivars adapted to Scandinavian cultivation areas. Derived cultivars ('Mari') were also used in breeding programs to breed for early flowering and adaptation to terminal drought in Mediterranean areas (Lundqvist 2009).

5.4 Flowering Time Genes and Floral Pathways in Barley

The genetic control of flowering time in response to photoperiod and vernalisation has been extensively studied in *Arabidopsis thaliana*, which is like barley and wheat, a facultative long-day plant and grows as a summer and winter annual. Flowering time genes and pathways as revealed in *Arabidopsis* show a high degree of conservation across plant species. Orthologs of a large number of *Arabidopsis* flowering time genes, notably from the photoperiod response pathway, have been detected in the cereals (Cockram et al. 2007; Distelfeld et al. 2009; Higgins et al. 2010). However, major flowering time genes in barley and wheat have been identified using natural genetic diversity and QTL mapping (Turner et al. 2005; Yan et al. 2003), rather than homology to *Arabidopsis* flowering time genes. Nevertheless, information from *Arabidopsis* has supported the functional characterisation of barley flowering time regulators and assignment to floral pathways. The major regulators of flowering time in barley are the photoperiod response gene *Ppd-H1* and the vernalisation responsive genes *VRN-H1*, *VRN-H2* and *VRN-H3* (Turner et al. 2005; Yan et al. 2003, 2004, 2006). Figure 5.2 provides an overview on barley flowering time genes and their connectivity within the flowering time pathway. Figure 5.3 indicates the map positions of major flowering time genes and QTL. Allelic variation and functional interactions between the genes are discussed below.

5.4.1 Photoperiod Pathway

The acquisition of day-length neutrality was crucial for the "green revolution" and the development of superior wheat cultivars (Borlaug 1983). Photoperiod

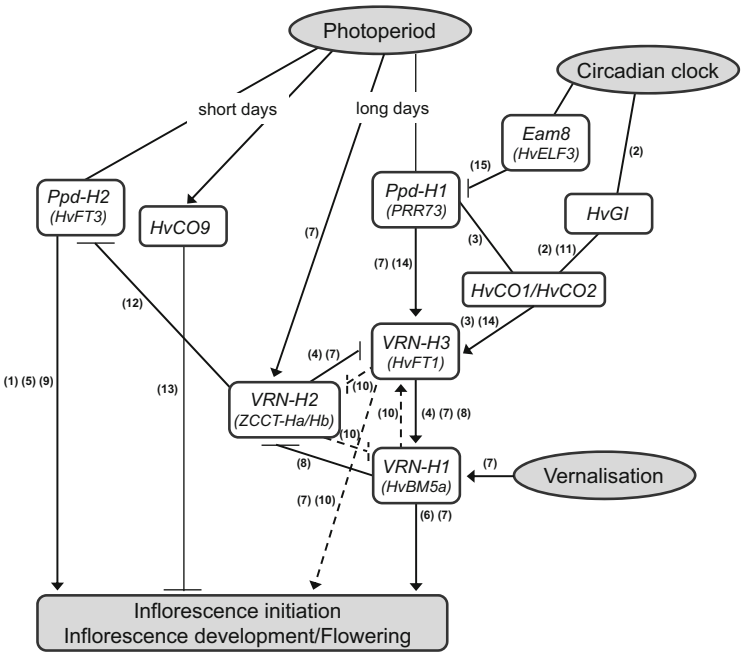

Fig. 5.2 Flowering time model in barley. The interactions between photoperiod and vernalisation pathways are shown. Numbers in *brackets* indicate literature in which experimental evidences support this model, and *dashed lines* indicate alternative models of gene interactions; (1) Laurie et al. (1995); (2) Dunford et al. (2005); (3) Turner et al. (2005); (4) Yan et al. (2006); (5) Faure et al. (2007); (6) Shitsukawa et al. (2007); (7) Hemming et al. (2008); (8) Li and Dubcovski (2008); (9) Kikuchi et al. (2009); (10) Shimada et al. (2009); (11) Shin-Young et al. (2010); (12) Casao et al. (2011); (13) Kikuchi et al. (2011); (14) Campoli et al. (2012)a; and (15) Faure et al. (2012)

insensitivity is widespread in the world's wheat varieties and predominates in regions where spring wheat is grown as a crop over the winter (short-day) period and where autumn-sown winter wheat needs to mature in the following year before the onset of high summer temperatures (Law 1987; Law and Worland 1997; Worland and Snape 2001). A mutation in the regulatory region of the photoperiod response factor Ppd-D1 was identified as causative for day-length neutrality in wheat (Beales et al. 2007). Recent studies have shown that functional variation at *Ppd-D1a*, *Ppd-A1a* or *Ppd-B1a* in tetraploid and hexaploid wheat are associated with deletions in the promoter of the gene or differences in copy number which all result in an upregulation of the *PPD1* homeologous genes (Wilhelm et al. 2009; Shaw et al. 2012; Diaz et al. 2012). In barley, the homologous photoperiod response gene *Ppd-H1* maps to the short arm of chromosome 2H and is considered the key gene in determining flowering time under long-day conditions. *Ppd-H1* is a member of the pseudo-response regulator (PRR) family, orthologous to the circadian clock gene *PRR7* in *Arabidopsis*. The dominant *Ppd-H1* allele causes early flowering under long days and is prevalent in (Mediterranean) winter and wild barley.

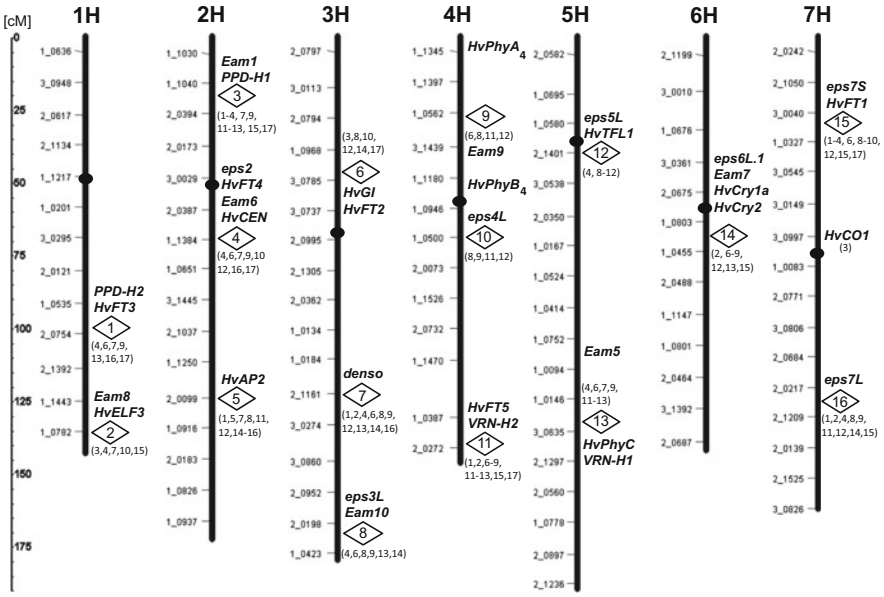

Fig. 5.3 Consensus map of QTL positions for flowering time in barley. Positions of QTL and flowering time candidate genes were projected onto Barley Consensus map of Muñoz-Amatriaín et al. (2011). Markers to the *left* of the chromosomes represent POPA SNP markers. Numbers in *diamond-shaped boxes* to the *right* of the chromosomes summarise approximate positions of QTL for flowering time. Positions of centromeres are indicated as *black ovals*. References for candidate genes are reported in the text. Publications corresponding to QTL positions are indicated with indices: (1) Baum et al. (2003); (2) Bezant et al. (1996); (3) Borràs-Gelonch et al. (2010); (4) Boyd et al. (2003); (5) Chen et al. (2009a); (6) Cuesta-Marcos et al. (2008a); (7) Cuesta-Marcos et al. (2008b); (8) Ivandic et al. (2002); (9) Laurie et al. (1995); (10) Marquez-Cedillo et al. (2001); (11) Pillen et al. (2003); (12) Pillen et al. (2004); (13) Szűcs et al. (2006); (14) Teulat et al. (2001); (15) von Korff et al. (2006); (16) von Korff et al. (2008); and (17) Wang et al. (2010)

A mutation in the conserved CCT domain of the gene causes late flowering under inductive long photoperiods, and this mutation has been selected in Northern European spring barley genotypes (Turner et al. 2005; Jones et al. 2008). Turner et al. (2005) have shown that barley genotypes with a dominant *Ppd-H1* allele are characterised by an elevated expression of *VRN-H3*/*HvFT1*. Similarly, increased expression of PPD1 in wheat upregulated the *TaFT* homeologous genes in a genome-independent manner (Shaw et al. 2012). *TaFT* and *VRN-H3* map to the short arm of the homeologous chromosome group 7 and encode a RAF kinase inhibitor with homology to the *Arabidopsis* gene *Flowering Locus T* (FT). Polymorphisms in the first intron of *VRN-H3* have been associated with winter or spring growth habit, where the spring allele shows a higher expression level (Yan et al. 2006). In *Arabidopsis*, FT is the mobile florigen hormone which moves as a protein from the leaves through the phloem to the shoot apical meristem where it induces the switch from vegetative to reproductive growth (Corbesier et al. 2007).

Tamaki et al. (2007) have shown that also in rice the protein encoded by *Hd3a*, orthologous to FT, moves from the leaf to the shoot apical meristem and induces flowering.

The prominent role of PPD1 in the control of photoperiod sensitivity in wheat and barley suggests that the circadian clock plays an important role in the control of flowering in cereals. Circadian clocks synchronise biological processes with the diurnal cycle, using molecular mechanisms that include interlocked transcriptional feedback loops. In *Arabidopsis*, the circadian clock is composed of three negative feedback loops: (a) the inhibition of evening complex (EC) genes *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*) and *LUX ARRHYTHMO* (*LUX*, also known as *PHYTOCLOCK1*) by the rise of CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) late at night, (b) the inhibition of *PRR* genes by the EC early at night, and (c) the inhibition of *LHY*/*CCA1* by TIMING OF CAB EXPRESSION1 (TOC1) in the morning (Huang et al. 2012; Pokhilko et al. 2012). In addition, the evening-expressed GIGANTEA (GI) protein was modelled as a negative regulator of the EC, which in turn inhibits *TOC1* expression (Pokhilko et al. 2012). Campoli et al. (2012b) have shown that circadian clock genes are structurally conserved in barley compared to *Arabidopsis*, and their circadian expression patterns suggested conserved functions. However, phylogenetic analyses revealed that duplications/deletions of clock genes occurred throughout the evolution of eudicots and monocots. For instance, the *PRR* genes duplicated independently in monocots and eudicots, and only one homologue of the two paralogous *Arabidopsis* clock genes *LHY*/*CCA1* is found in monocots (Takata et al. 2010; Campoli et al. 2012b). In this context it is interesting to note that natural variation at *PPD1* in barley and wheat are major determinants of photoperiod sensitivity (Turner et al. 2005; Beales et al. 2007), while natural variation at *PRR* genes in *Arabidopsis* did not have a strong effect on flowering time (Ehrenreich et al. 2009). In barley, day-length neutrality has not been widely used in breeding programs, but natural and induced *early maturity* (*eam*) mutants have been used to breed for early flowering spring barley (Lundqvist 2009). Recently, the gene underlying the *eam8* locus on chromosome 1H was identified as *HvELF3*, orthologous to the *Arabidopsis* clock gene *ELF3* (Faure et al. 2012; Zakhrabekova et al. 2012). Faure et al. (2012) showed that under noninductive short-day conditions, the mutation at *HvELF3* causes an upregulation of *Ppd-H1* and consequent activation of the downstream photoperiodic pathway. In *Arabidopsis*, ELF3 physically associates with the promoter of *PRR9* to repress its transcription suggesting that transcriptional targets of ELF3 are partly conserved between *Arabidopsis* and barley (Dixon et al. 2011; Herrero et al. 2012). The molecular and phenotypic effects of the mutation in *HvELF3* were thus similar to the effects of a mutation in the promoter of *Ppd-D1a*; both mutations cause an upregulation of *PPD1* and photoperiod insensitivity.

The circadian clock also controls the expression of output genes from the flowering time pathway. In *Arabidopsis*, *FT* expression is triggered by the photoperiod response gene *CONSTANS* (*CO*) (Samach et al. 2000). *CO* is regulated at the transcriptional level by several genes that are part of the circadian clock or are

under circadian clock control, so that *CO* mRNA accumulates at the end of the day. At the protein level, CO is regulated by the cryptochromes cry1 and cry2, the phytochromes phyA and phyB, and the ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) that respectively stabilise CO in light or destabilise CO in darkness (Jang et al. 2008). As *CO* transcription occurs before dusk in long days but after dusk in short days, CO protein only accumulates and mediates the transcription of *FT* under long days (Turck et al. 2008).

In barley, nine orthologs of the *AtCO* gene have been isolated, with *HvCO1* and *HvCO2* showing the highest similarity to the *Arabidopsis CO* gene, while *HvCO1* is the positional ortholog of *Hd1*, a major determinant of photoperiod sensitivity in rice (Griffiths et al. 2003; Higgins et al. 2010). Turner et al. (2005) suggested that the mutation in *Ppd-H1* of spring barley delayed flowering time by shifting the diurnal expression peaks of *HvCO1* and *HvCO2* mRNA into the dark phase, so that the protein is not synthesised and *VRN-H3/HvFT1* not expressed. Campoli et al. (2012a) have recently confirmed that HvCO1 induces flowering in barley, and overexpression of *HvCO1* upregulated *HvFT1* and accelerated flowering under long- and short-day conditions. However, analysis of a mapping population segregating for the overexpression of *HvCO1* and functional variation at *Ppd-H1* showed that *Ppd-H1* induced *HvFT1* expression downstream of *HvCO1* transcription (Campoli et al. 2012a).

In *Arabidopsis*, *CO* transcription is controlled by the clock protein GI (Fowler et al. 1999). In barley, functional conservation of the single ortholog *HvGI* has not yet been demonstrated (Dunford et al. 2005). However, in rice, overexpression of *OsGI* induced the expression of *Hd1*, the rice ortholog of *CO* in *Arabidopsis* (Hayama et al. 2003). In addition, heterologous expression of the *Brachypodium distachyon* GIGANTEA protein in a GI-deficient *Arabidopsis* mutant rescued the late flowering phenotype, suggesting that the role of GI is conserved in the Triticeae species (Shin-Young et al. 2010).

In barley, five different *FT*-like genes were detected, *HvFT1*, *HvFT2*, *HvFT3*, *HvFT4* and *HvFT5* (Faure et al. 2007), of which only *HvFT1* (*VRN-H3*) has been identified as a flowering promoter (Kikuchi et al. 2009). However, *HvFT3* has been recently proposed as a candidate gene for the photoperiod response gene *Ppd-H2* which maps to the long arm of chromosome 1H (Faure et al. 2007; Kikuchi et al. 2009). So far, two major functional variants of *HvFT3* are known (Casao et al. 2011; Cuesta-Marcos et al. 2008a). The dominant functional allele is prevalent in southern European barley germplasm and causes faster flowering under short-day conditions when vernalisation is not fully satisfied (Casao et al. 2011). A partial deletion of the gene results in a recessive nonfunctional allele that is common in winter barley (Kikuchi et al. 2009; Faure et al. 2007). The expression of both *HvFT1* and *HvFT3* is repressed by *VRN-H2* and thus also controlled by the vernalisation pathway (Yan et al. 2006; Casao et al. 2011).

5.4.2 Vernalisation Pathway

Vernalisation response in barley is primarily controlled by genetic variations at *VRN-H1* and *VRN-H2*. *VRN-H1*, located on the long arm of chromosome 5H, encodes a MADS-box transcription factor with a high similarity to the *Arabidopsis* meristem identity genes *APETALA1*, *CAULIFLOWER* and *FRUITFUL* (Yan et al. 2003). The recessive winter allele at *VRN-H1* is only expressed after exposure to cold. Insertions or deletions in the first intron of *VRN-H1* in spring barley cause an upregulation of the gene independently of vernalisation (Hemming et al. 2009). Hemming et al. (2009) have identified regions within the first intron of *VRN-H1* associated with repression of the gene in non-vernalised plants. These regions, however, are not required for the induction of *VRN-H1* by cold (Hemming et al. 2009). Expression of *VRN-H1* is important for the transition to reproductive growth. The *Triticum monococcum* mutant *mvp* (*maintained vegetative phase*), which carries a deletion of the *VRN1* locus, never transitioned from the vegetative to the reproductive phase (Shitsukawa et al. 2007). Shimada et al. (2009) described that in wheat, the upregulation of *VRN1* under long days was followed by the accumulation of *VRN3* (*TaFT*) transcripts, while *TaFT* was not expressed in the *mvp* mutant of einkorn wheat. Consequently, the authors suggested that *VRN1* is upstream of *VRN3* (*FT*) and upregulates *VRN3* expression under long-day conditions. Diaz et al. (2012) have demonstrated that as for PPD1, copy number variation of VRN1 correlated with the expression level and vernalisation requirement.

In barley, *VRN-H1* downregulates expression of *VRN-H2*, which is only expressed under long-day conditions. The *VRN-H2* region on chromosome 4HL includes one truncated and two full sequence ZCCT (Zinc finger and CCT domain) genes, *ZCCT-Ha*, *ZCCT-Hb* and *ZCCT-Hc* with no clear orthologs in *Arabidopsis* (Yan et al. 2004). In photoperiod-sensitive winter barley, *VRN-H2* represses *VRN-H3* (*HvFT1*) to counteract the *Ppd-H1*-dependent long-day induction of *VRN-H3* prior to winter. *VRN-H2* expression is maintained at high levels, prior to vernalisation, and downregulated by *VRN-H1* during exposure to cold. Upregulation of *VRN-H1* during vernalisation and consequent downregulation of *VRN-H2* promote inflorescence meristem identity at the shoot apex and accelerate inflorescence initiation. Downregulation of *VRN-H2* transcript levels in the leaves facilitates the upregulation of *VRN-H3* during long days mediated by *Ppd-H1* and possibly by *HvCO1* (Yan et al. 2006; Hemming et al. 2008; Campoli et al. 2012a). High levels of *VRN-H3* in turn upregulate *VRN-H1*. Li and Dubcovski (2008) have shown that wheat *VRN3* induces *VRN1* transcription via the interaction with FDL2 (FD-LIKE2) and argue that *VRN3* is the integrator of low-temperature and long day-length responses.

Kikuchi et al. (2011) have recently shown that *HvCO9*, which belongs to the same grass-specific *CO*-like subfamily of the flowering repressors *VRN-H2* in barley and *Ghd7* in rice (Xue et al. 2008), delays flowering under noninductive short-day conditions, possibly by downregulating *HvFT1*. In the Triticeae, the chromosomal region on 4H containing the *Vrn2* locus has originated from a

duplication of a chromosomal region on chromosome 1 carrying the *HvCO9* locus (Cockram et al. 2010). The *Vrn2* locus may thus be derived from a targeted duplication of *HvCO9* to the homologous region after the divergence of Triticeae (Kikuchi et al. 2011). It is interesting that grass species have developed systems for flowering repression that are different from those of *Arabidopsis*. Despite the homology between *Arabidopsis* and cereal flowering time genes, gene duplication may have favoured functional diversification of flowering time pathways. Functional comparison of cereal and *Arabidopsis CO* and *FT* families, for example, demonstrates that their connectivity within the flowering pathways has been modified; and they can be regulated by different external and internal factors.

5.5 QTLs for Flowering Time in Barley

Functional variation at *Ppd-H1*, *Ppd-H2*, *VRN-H1*, *VRN-H2* and *VRN-H3* has been consistently identified in QTL studies using crosses between elite winter and spring barley genotypes (Laurie et al. 1995; Sameri et al. 2011). However, QTL studies within winter barley germplasm, primarily in Mediterranean barley including wild barley (*H. spontaneum*) and barley landraces, have revealed additional major flowering time loci. Figure 5.3 shows consensus QTLs for flowering time in barley and indicates candidate genes or potentially allelic mutants which map close to these QTLs. A selection of the consensus QTLs and possible candidate genes are discussed below. SSR markers linked to these QTLs are given in Table 5.1 and may be used in QTL studies for flowering time control or marker-assisted selection in barley.

In crosses involving wild barley or Mediterranean landrace genotypes, QTLs for flowering time are consistently detected close to the *eam6* locus at the centromeric region of chromosome 2H (Marquez-Cedillo et al. 2001; Pillen et al. 2004; von Korff et al. 2008; Wang et al. 2010). This locus has major effects on flowering time in autumn-sown field trials in Mediterranean and Australian environments and has been associated with variation in the duration of the basic vegetative period (Boyd et al. 2003; Cuesta-Marcos et al. 2008a, b). *Eam6* on chromosome 2H was identified as an ortholog of Antirrhinum *CENTRORADIALIS* (*HvCEN*), homologous to *Arabidopsis TFL1*. *TFL1* is an *FT*-like gene but unlike *FT* encodes a repressor of flowering. Comadran et al. (2012) showed that natural variation at *HvCEN* contributed to the adaptation of barley to higher latitudes with cool and wet summers and thus extended growing seasons. Genetic variation for flowering time control was also identified at the *FLT-2L* locus on the long arm of chromosome 2H (Teulat et al. 2001; Ivandic et al. 2002; Boyd et al. 2003; Baum et al. 2003; Pillen et al. 2003, 2004; von Korff et al. 2006, 2008, 2010; Eleuch et al. 2008; Borràs-Gelonch et al. 2010). The locus, which also affected plant height and rachis internode length, was fine mapped to a region which included *HvAP2*, a gene encoding an AP2 domain protein, with sequence similarity to the wheat domestication gene *Q* located on chromosome 5A and conferring a similar phenotype to the

Table 5.1 Marker assays for major flowering time genes and QTLs in barley

Locus/gene	Accession number	Allele/ marker	Chr	Bin	Forward primer	Reverse primer	Marker assay	Polymorphism	References
Genes									
Ppd-H1	AY970701	*Ppd-H1*/ *ppd-H1*	2HS	4–5	CCAGTGTTGTCAATCCTTCG	GCTCCCGTTATTGGTGTTGT	SNP/ HRM	G/T	Turner et al. (2005)
Ppd-H2 (HvFT3)	AB476614	*Ppd-H2*/ *ppd-H2*	1HL	14	CCGTTCCATTAAGGATAGCC	CCATCCGGTGGATACCAG	INDEL	1,163 bp/ no fragment	Faure et al. (2007)
VRN-H2	DQ492699	*VRN-H2*/ *vrn-H2*	4HL	13	CCTAGTTAAAACATATATCCATAGAGC	GATCGTTGCGTTGCTAATAGTG	INDEL	ZCCT-Ha: 307 bp	Karsai et al. (2005)
	DQ492698							ZCCT-Hb: 273 bp/ no fragment	
VRN-H1 (spring allele)	AY750993	*HvVRN1*	5HL	11	TTCATCATGGATCGCCAGTA	AAAGCTCCTGCCAACTACGA	STS	403 bp	Hemming et al. (2009)
vrn-H1 (winter allele)	AY750993	*HvVRN1-1*	5HL	11	GCTCCAGCTGATGAAACTCC	CTTCATGGTTTTGCAAGCTCC	STS	477 bp	Hemming et al. (2009)
VRN-H3 (*HvFT1*)	DQ898515	*VRN-H3*/ *vrn-H3*	7HS	13	TTGATCATGATTTTTCTATGCTTTC	AGTCATGTGATTGCAGAAGCTC	SNP/ HRM	A/T	Yan et al. (2006)
QTL									
Eam8		GBMS12	1HL	14	GCTGATGATGCGCATGAG	TTGCTTCCCTTGCCTCTCTA	SSR	(CT)11(CA)20	Laurie et al. (1995)
Eam6		Bmac0684	2H	8	TTCCGTTGAGCTTTCATACAC	ATTGAATCCCAACAGACACAA	SSR	(TA)7(TG)11, (TG)11(TTTG)5	Laurie et al. (1995)
Flt-2L/ *HvAP2*		EBmac0415	2HL	13	GAAACCCATCATAGCAGC	AAACAGCAGCAAGAGGAG	SSR	(AC)17	Chen et al. (2009b)
sdw1/*Ga20ox*		Bmag0013	3HL	10	AAGGGGAATCAAAATGGGAG	TCGAATAGGTCTCCGAAGAAA	SSR	(CT)21	Jia et al. (2009)
Eam10/ *eps3.L*		HVM62	3HL	14	TCGCGACCAGACGAGAAG	AGCTAGCCGACGACGCAC	SSR	(GA)11	Laurie et al. (1995)
Eam7/ *eps6L.1*		HVM74	6HS	6	AGGAAGTCATTGCGTGAG	TGATCAAGAATGATAACATGG	SSR	(GA)13	Laurie et al. (1995)
eps7S		Bmag206	7HS	3	TTTTCCCCTATTATAGTGACG	TAGAACTGGGTATTTCCTTGA	SSR	(GT)5(AG)14	Laurie et al. (1995)
eps7L		BMS64	7HL	8	CTGCAGGTTTCAGGAAGG	AGATGCCCGCAAAGAGTT	SSR	(AC)21	Laurie et al. (1995)

barley *Flt-2L* mutation(Chen et al. 2009a). A number of crosses involving elite and exotic germplasm also revealed genetic variation for flowering time at the long arm of chromosome 3H (Laurie et al. 1995; Bezant et al. 1996; Boyd et al. 2003; Baum et al. 2003; Szűcs et al. 2006; Cuesta-Marcos et al. 2008a). Early flowering at this locus was caused by the exotic allele and was correlated with increased plant height and reduced yield under favourable conditions, but increased yield under marginal rain-fed conditions (von Korff et al. 2006, 2008). This QTL coincides with the *sdw1*/*denso* locus which reduces growth and has been selected in elite barley to reduce lodging and optimise yield under favourable conditions. *Ga20-oxidase*, a gene involved in the synthesis of gibberellin, has been recently proposed as a potential candidate for this locus (Jia et al. 2009).

QTLs for flowering time at the centromeric region of chromosome 6H also coincided with QTLs for plant height and yield, where the wild barley alleles reduced time to flowering, plant height and yield under favourable conditions (Laurie et al. 1995; Bezant et al. 1996; Ivandic et al. 2002; Pillen et al. 2004; von Korff et al. 2006; Cuesta-Marcos et al. 2008a, b). The blue/UV-A light cryptochrome photoreceptors Cry1a and Cry2 which regulate plant growth and development (Quail 2002) map to the centromeric region of 6H (Szűcs et al. 2006). Furthermore, the same region of 6H harbours the *eam7* mutation which determines photoperiod insensitivity and early flowering under long-day conditions (Stracke and Börner 1998). QTL studies for agronomic traits suggest that flowering time is strongly correlated with plant height and yield. However, very little is known about the direct or indirect effects of individual flowering time genes and QTLs on plant architecture and yield structure. Genetic dissection of individual meristem phases may thus allow further characterising pleiotropic effects of individual flowering time genes on plant architecture and yield components.

5.6 Pleiotropic Effects of Flowering Time Genes

Studies in rice and tomato have already demonstrated that flowering time genes have pleiotropic effects on a number of traits including inflorescence architecture and grain yield. In rice, *Ghd7* encoding a CCT domain protein acts as a regulator of flowering time, panicle size and seed number (Xue et al. 2008). In tomato, the loss-of-function allele of *single flower truss* (*SFT*) increases the total number of inflorescences, flowers and fruits per plant. This gene was shown to increase yield by up to 60 % if in heterozygous state, providing one of the first examples of overdominance in heterosis for yield (Krieger et al. 2010). Although major cereal genes have been identified which affect the time from germination to flowering/anthesis, little information exists about genes and molecular changes in the leaf and in the meristem that determine the initiation of the different developmental phases (Shitsukawa et al. 2007; Chen et al. 2009b; González et al. 2005). In wheat, expression of *VRN1* is important for the transition to a reproductive meristem (Shitsukawa et al. 2007). However, Chen et al. (2009b) found that variation in

stem elongation and inflorescence development mapped close to *VRN-H1* in a barley mapping population, suggesting that this gene also affects later developmental phases. Variation in the duration of the vegetative phase was also ascribed to *eam* or *eps* loci. Lewis et al. (2008) found that variation at the *eps-A1* locus affected the transition to the reproductive stage and formation of a terminal spikelet, but not inflorescence development in wheat. These differences were paralleled by a significant decrease in the number of spikelets per spike, in both greenhouse and field experiments. In contrast, variation at the photoperiod response gene *Ppd-H1* and overexpression of *HvCO1* primarily affected the stem elongation phase and inflorescence development (Campoli et al. 2012a). However, studies in wheat have shown that variation at *Ppd-D1* affected all meristem phases (González et al. 2005). The authors also showed that lengthening the late reproductive phase of stem elongation in wheat increased spike weight and the number of fertile florets at anthesis. These studies demonstrate that flowering time genes have an indirect effect on yield potential by fine-tuning flowering time for an optimal adaptation to different environments. In addition to this indirect effect, flowering time genes have a more direct impact on yield by affecting basic developmental processes and individual grain yield components. Understanding the genetic basis of pre-anthesis development may thus contribute to unravelling the genetic basis of inflorescence architectures and thus yield in cereals.

References

Baum M, Grando S, Backes G, Jahoor A, Sabbagh A, Ceccarelli S (2003) QTLs for agronomic traits in the Mediterranean environment identified in recombinant inbred lines of the cross 'Arta' x *H. spontaneum* 41-1. Theor Appl Genet 107(7):1215–1225

Beales J, Turner A, Griffiths S, Snape JW, Laurie DA (2007) A pseudo-response regulator is misexpressed in the photoperiod insensitive Ppd-D1a mutant of wheat (Triticum aestivum L.). Theor Appl Genet 115:721–733

Bezant J, Laurie D, Pratchett N, Chojecki J, Kearsey M (1996) Marker regression mapping of QTL controlling flowering time and plant height in a spring barley (*Hordeum vulgare* L.) cross. Heredity 77:64–73

Borlaug NE (1983) Contributions of conventional plant breeding to food production. Science 219:689–693

Borràs-Gelonch G, Slafer GA, Casas AM, van Eeuwijk F, Romagosa I (2010) Genetic control of pre-heading phases and other traits related to development in a double-haploid barley (*Hordeum vulgare L.*) population. Field Crops Res 119(1):36–47

Boyd WJR, Li CD, Grime CR, Cakir M, Potipibool S, Kaveeta L, Men S, Kamali MRJ, Barr AR, Moody DB, Lance RCM, Logue SJ, Raman H, Read BJ (2003) Conventional and molecular genetic analysis of factors contributing to variation in the timing of heading among spring barley (*Hordeum vulgare* L.) genotypes grown over a mild winter growing season. Aust J Agric Res 54(12):1277–1301

Campoli C, Drosse B, Searle I, Coupland G, von Korff M (2012a) Functional characterisation of *HvCO1*, the barley (*Hordeum vulgare*) flowering time ortholog of CONSTANS. Plant J 69:868–880

Campoli C, Shtaya M, Davis SJ, von Korff M (2012b) Expression conservation within the circadian clock of a monocot: natural variation at barley *Ppd-H1* affects circadian expression of flowering time genes, but not clock orthologs. BMC Plant Biol 12:97

Casao MC, Iguarta E, Karsai I, Lasa JM, Gracia MP, Casas AM (2011) Expression analysis of vernalization and day-length response genes in barley (Hordeum vulgare L.) indicates that VRNH2 is a repressor of PPDH2 (HvFT3) under long days. J Exp Bot 62:1939–1949

Chen A, Baumann U, Fincher GB, Collins NC (2009a) Flt-2L, a locus in barley controlling flowering time, spike density, and plant height. Funct Integr Genomics 9(2):243–254

Chen Y, Carver B, Wang S, Zhang F, Yan L (2009b) Genetic loci associated with stem elongation and winter dormancy release in wheat. Theor Appl Genet 118(5):881–889

Cockram J, Jones H, Leigh FJ, O'Sullivan D, Powell W, Laurie DA, Greenland AJ (2007) Control of flowering time in temperate cereals: genes, domestication, and sustainable productivity. J Exp Bot 58:1234–1244

Cockram J, Howells RM, O'Sullivan DM (2010) Segmental chromosomal duplications harbouring group IV CONSTANS-like genes in cereals. Genome 53:231–240

Comadran J, Kilian B, Russell J, Ramsay L, Stein N, Ganal M, Shaw P, Bayer M, Thomas W, Marshall D, Hedley P, Tondelli A, Pecchioni N, Francia E, Korzun V, Walther A, Waugh R (2012) Natural variation in a homolog of *Antirrhinum CENTRORADIALIS* contributed to spring growth habit and environmental adaptation in cultivated barley. Nat Genet 44:1388–1392

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science 316:1030–1033

Cuesta-Marcos A, Casas A, Yahiaoui S, Gracia M, Lasa J, Igartua E (2008a) Joint analysis for heading date QTL in small interconnected barley populations. Mol Breed 21(3):383–399

Cuesta-Marcos A, Igartua E, Ciudad F, Codesal P, Russell J, Molina-Cano J, Moralejo M, Szűcs P, Gracia M, Lasa J, Casas A (2008b) Heading date QTL in a spring × winter barley cross evaluated in Mediterranean environments. Mol Breed 21(4):455–471

Diaz A, Zikhali M, Turner AS, Isaac P, Laurie DA (2012) Copy number variation affecting the *Photoperiod-B1* and *Vernalization-A1* genes is associated with altered flowering time in wheat (*Triticum aestivum*). PLoS One 7(3):e33234. doi:10.1371/journal.pone.0033234

Distelfeld A, Li C, Dubcovsky J (2009) Regulation of flowering in temperate cereals. Curr Opin Plant Biol 12:178–184

Dixon LE, Knox K, Kozma-Bogmar L, Southern MM, Pokhilko A, Millar AJ (2011) Temporal repression of core circadian genes is mediated through EARLY FLOWERING 3 in Arabidopsis. Curr Biol 21:120–125

Dunford RP, Griffiths S, Christodoulou V, Laurie DA (2005) Characterisation of a barley (Hordeum vulgare L.) homologue of the *Arabidopsis* flowering time regulator GIGANTEA. Theor Appl Genet 110:925–931

Ehrenreich I, Hanzawa Y, Chou L, Roe J, Kover PX et al (2009) Candidate gene association mapping of Arabidopsis flowering time. Genetics 183:325–335. doi:10.1534/genetics.109.105189

Eleuch L, Abderrazek J, Grando S, Ceccarelli S, von Korff M, Hajer A, Daaloul A, Baum M (2008) Genetic diversity and association analysis for salinity tolerance, heading date and plant height of barley germplasm using SSR markers. J Integr Plant Biol 50:1004–1014

Ellis RH, Roberts EH, Summerfield RJ, Cooper JP (1988) Environmental control of flowering in barley (Hordeum vulgare L.). II. Rate of development as a function of temperature and photoperiod and its modification by low-temperature vernalization. Ann Bot 62(2):145–158

Ellis RH, Summerfield RJ, Roberts EH, Cooper JP (1989) Environmental control of flowering in barley (*Hordeum vulgare*). III. Analysis of potential vernalization responses, and methods of screening germplasm for sensitivity to photoperiod and temperature. Ann Bot 63(6):687–704

Faure S, Higgins J, Turner A, Laurie DA (2007) The FLOWERING LOCUS T-like gene family in barley (*Hordeum vulgare*). Genetics 176:599–609

Faure S, Turner AS, Gruszka D, Christodoulou V, Davis SJ, von Korff M, Laurie DA (2012) Mutation at the circadian clock gene EARLY MATURITY 8 adapts domesticated barley (*Hordeum vulgare*) to short growing seasons. Proc Natl Acad Sci U S A 109:8328–8333

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J (1999) GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. EMBO J 18:4679–4688

Ghiglione HO, Gonzalez FG, Serrago R, Maldonado SB, Chilcott C, Cura JA, Miralles DJ, Zhu T, Casal JJ (2008) Autophagy regulated by day length determines the number of fertile florets in wheat. Plant J 55:1010–1024

González FG, Slafer GA, Miralles DJ (2002) Vernalization and photoperiod responses in wheat pre-flowering reproductive phases. Field Crops Res 74(2–3):183–195

González FG, Slafer GA, Miralles DJ (2003) Floret development and spike growth as affected by photoperiod during stem elongation in wheat. Field Crops Res 81(1):29–38

González FG, Slafer GA, Miralles DJ (2005) Pre-anthesis development and number of fertile florets in wheat as affected by photoperiod sensitivity genes Ppd-D1 and Ppd-B1. Euphytica 146(3):253–269

González FG, Miralles DJ, Slafer GA (2011) Wheat floret survival as related to pre-anthesis spike growth. J Exp Bot 62(14):4889–4901

Griffiths FEW, Lyndon RF, Bennett MD (1985) The effects of vernalization on the growth of the wheat shoot apex. Ann Bot 56(4):501–511

Griffiths S, Dunford RP, Coupland G, Laurie DA (2003) The evolution of *CONSTANS*-like gene families in barley, rice, and *Arabidopsis*. Plant Physiol 131:1855–1867

Hayama R, Yokoi A, Tamaki S, Yano M, Shimamoto K (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. Nature 422:719–722

Hemming MN, Peacock WJ, Dennis ES, Trevaskis B (2008) Low-temperature and daylength cues are integrated to regulate FLOWERING LOCUS T in barley. Plant Physiol 147:355–366

Hemming MN, Fieg S, Peacock WJ, Dennis ES, Trevaskis B (2009) Regions associated with repression of the barley (Hordeum vulgare) VERNALIZATION1 gene are not required for cold induction. Mol Genet Genomics 282:107–117

Herrero E, Kolmos E, Bujdoso N, Yuan Y, Wang M, Berns MC, Uhlworm H, Coupland G, Saini R, Jaskolski M, Webb A, Gonçalves J, Davis SJ (2012) EARLY FLOWERING4 recruitment of EARLY FLOWERING3 in the nucleus sustains the Arabidopsis circadian clock. Plant Cell 24:428–443

Higgins JA, Bailey PC, Laurie DA (2010) Comparative genomics of flowering time pathways using Brachypodium distachyon as a model for the temperate grasses. PLoS One 5:e10065

Huang W, Pérez-García P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P (2012) Mapping the core of the Arabidopsis circadian clock defines the network structure of the oscillator. Science 336:75–79

Ivandic V, Hackett CA, Nevo E, Keith R, Thomas WT, Forster BP (2002) Analysis of simple sequence repeats (SSRs) in wild barley from the Fertile Crescent: associations with ecology, geography and flowering time. Plant Mol Biol 48(5–6):511–527

Jang S, Marchal V, Pahigrahi KCS, Wenkel S, Soppe W, Deng XW, Valverde F, Coupland G (2008) *Arabidopsis* COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J 27:1277–1288

Jia Q, Zhang J, Westcott S, Zhang X, Bellgard M, Lance R, Li C (2009) *GA-20 oxidase* as a candidate for the semidwarf gene sdw1/denso in barley. Funct Integr Genomics 9:255–262

Jones H, Leigh FJ, Mackay I, Bower MA, Smith LMJ, Charles MP, Jones G, Jones MK, Brown TA, Powell W (2008) Population-based resequencing reveals that the flowering time adaptation of cultivated barley originated east of the Fertile Crescent. Mol Biol Evol 25:2211–2219

Karsai I, Szucs P, Mészáros K, Filichkina T, Hayes PM, Skinner JS, Láng L, Bedö Z (2005) The Vrn-H2 locus is a major determinant of flowering time in a facultative winter growth habit barley (*Hordeum vulgare* L.) mapping population. Theor Appl Genet 110:1458–1466

Kikuchi R, Kawahigashi H, Ando T, Tonooka T, Handa H (2009) Molecular and functional characterization of PEBP genes in barley reveal the diversification of their roles in flowering. Plant Physiol 149:1341–1353

Kikuchi R, Kawahigashi H, Oshima M, Ando T, Handa H (2011) The differential expression of HvCO9, a member of the CONSTANS-like gene family, contributes to the control of flowering under short-day conditions in barley. J Exp Bot 63(2):773–784

Krieger U, Lippman ZB, Zamir D (2010) The flowering gene *SINGLE FLOWER TRUSS* drives heterosis for yield in tomato. Nat Genet 42:459–463

Laurie DA, Pratchett N, Snape JW, Bezant JH (1995) RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter × spring barley (*Hordeum vulgare* L.) cross. Genome 38(3):575–585

Law CN (1987) The genetic control of day-length response in wheat. In: Atherton JE (ed) Manipulation of xowering. Butterworths, London, pp 225–240

Law CN, Worland AJ (1997) Genetic analysis of some flowering time and adaptive traits in wheat. New Phytol 137:19–28

Lewis S, Faricelli ME, Appendino ML, Valárik M, Dubcovsky J (2008) The chromosome region including the earliness per se locus Eps-Am1 affects the duration of early developmental phases and spikelet number in diploid wheat. J Exp Bot 59(13):3595–3607

Li C, Dubcovski J (2008) Wheat FT protein regulates VRN1 transcription through interactions with FDL2. Plant J 55:543–554

Lundqvist U (2009) Eighty years of Scandinavian barley mutation genetics and breeding. In: Shu QY (ed) Induced mutations in the genomics era. FAO, Rome, pp 39–43

Marquez-Cedillo LA, Hayes PM, Kleinhofs A, Legge WG, Rossnagel BG, Sato K, Ullrich SE, Wesenberg DM, TNABGM Project (2001) QTL analysis of agronomic traits in barley based on the doubled haploid progeny of two elite North American varieties representing different germplasm groups. Theor Appl Genet 103:625–637

Miralles DJ, Richards RA (2000) Responses of leaf and tiller emergence and primordium initiation in wheat and barley to interchanged photoperiod. Ann Bot 85:655–663

Muñoz-Amatriaín M, Moscou MJ, Bhat PR, Svensson JT, Bartoš J, Suchánková P, Šimková H, Endo TR, Fenton RD, Lonardi S, Castillo AM, Chao S, Cistué L, Cuesta-Marcos A, Forrest KL, Hayden MJ, Hayes PM, Horsley RD, Makoto K, Moody D, Sato K, Vallés MP, Wulff BBH, Muehlbauer GJ, Doležel J, Close TJ (2011) An improved consensus linkage map of barley based on flow-sorted chromosomes and single nucleotide polymorphism markers. Plant Genome 4(3):238–249

Pillen K, Zacharias A, Leon J (2003) Advanced backcross QTL analysis in barley (*Hordeum vulgare* L.). Theor Appl Genet 107:340–352

Pillen K, Zacharias A, Leon J (2004) Comparative AB-QTL analysis in barley using a single exotic donor of *Hordeum vulgare* ssp. *spontaneum*. Theor Appl Genet 108(8):1591–1601

Pokhilko A, Fernández AP, Edwards KD, Southern MM, Halliday KJ, Millar AJ (2012) The clock gene circuit in Arabidopsis includes a repressilator with additional feedback loops. Mol Syst Biol 8:574

Quail PH (2002) Photosensory perception and signalling in plant cells: new paradigms? Curr Opin Plant Biol 14:180–188

Reynolds M, Foulkes MJ, Slafer GA, Berry P, Parry MAJ, Snape JW, Angus WJ (2009) Raising yield potential in wheat. J Exp Bot 60(7):1899–1918

Roberts EH, Summerfield RJ, Cooper JP, Ellis RH (1988) Environmental control of flowering in barley (*Hordeum vulgare* L.). I. Photoperiod limits to long-day responses, photoperiod-insensitive phases and effects of low-temperature and short-day vernalization. Ann Bot 62 (2):127–144

Rollins JA, Drosse B, Mulki MA, Grando S, Baum M, Singh M, Ceccarelli S, von Korff M (2013) Variation at the vernalisation genes Vrn-H1 and Vrn-H2 determines growth and yield stability in barley (*Hordeum vulgare*) grown under dryland conditions in Syria. Theor Appl Genet 126 (11):2803–2824

Saisho D, Ishii M, Hori K, Sato K (2011) Natural variation of barley vernalization requirements: implication of quantitative variation of winter growth habit as an adaptive trait in East Asia. Plant Cell Physiol 2011(52):724–727

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanogsky MF, Coupland G (2000) Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis*. Science 288:1613–1616

Sameri M, Pourkheirandish M, Chen G, Takuji Tonooka T, Komatsuda T (2011) Detection of photoperiod responsive and non-responsive flowering time QTL in barley. Breed Sci 61:183–188

Shaw LM, Turner AS, Laurie DA (2012) The impact of photoperiod insensitive *Ppd-1a* mutations on the photoperiod pathway across the three genomes of hexaploid wheat (*Triticum aestivum*). Plant J 71:71–84

Shimada S, Ogawa T, Kitagawa S, Suzuki T, Ikari C, Shitsukawa N, Abe T, Kawahigashi H, Kikuchi R, Handa H, Murai K (2009) A genetic network of flowering-time genes in wheat leaves, in which an APETALA1/FRUITFUL-like gene, VRN1, is upstream of FLOWERING LOCUS T. Plant J 58:668–681

Shin-Young H, Sangmin L, Pil JS, Moon-Sik Y, Chung-Mo P (2010) Identification and molecular characterization of a Brachypodium distachyon GIGANTEA gene: functional conservation in monocot and dicot plants. Plant Mol Biol 72:485–497

Shitsukawa N, Ikari C, Shimada S, Kitagawa S, Sakamoto K, Saito H, Ryuto H, Fukumishi N, Abe T, Takumi S, Nasuda S, Murai K (2007) The einkorn wheat (Triticum monococcum) mutant, maintained vegetative phase, is caused by a deletion in the VRN1 gene. Genes Genet Syst 82:167–170

Slafer GA (2003) Genetic basis of yield as viewed from a crop physiologists perspective. Ann Appl Biol 142:117–128

Slafer GA, Rawson HM (1994) Sensitivity of wheat phasic development to major environmental factors: a re-examination of some assumptions made by physiologists and modellers. Aust J Plant Physiol 21:393–426

Slafer GA, Abeledo LG, Miralles DJ, Gonzalez FG, Whitechurch EM (2001) Photoperiod sensitivity during stem elongation as an avenue to raise potential yield in wheat. Euphytica 119:191–197

Stracke S, Börner A (1998) Molecular mapping of the photoperiod response gene ea7 in barley. Theor Appl Genet 97:797–800

Szűcs P, Karsai I, von Zitzewitz J, Mészáros K, Cooper LL, Gu YQ, Chen TH, Hayes PM, Skinner JS (2006) Positional relationships between photoperiod response QTL and photoreceptor and vernalization genes in barley. Theor Appl Genet 112:1277–1285

Takata N, Saito S, Saito CT, Uemura M (2010) Phylogenetic footprint of the plant clock system in angiosperms: evolutionary processes of pseudo-response regulators. BMC Evol Biol 10:126

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. Science 316:1033–1036

Teulat B, Merah O, Souyris I, This D (2001) QTLs for agronomic traits from a Mediterranean barley progeny grown in several environments. Theor Appl Genet 103:774–787

Turck F, Fornara F, Coupland G (2008) Regulation and identity of Florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol 59:573–594

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. Science 310:1031–1034

von Korff M, Wang H, Leon J, Pillen K (2006) AB-QTL analysis in spring barley. II: Detection of favourable exotic alleles for agronomic traits introgressed from wild barley (*H. vulgare* ssp. *spontaneum*). Theor Appl Genet 112(7):1221–1231

von Korff M, Grando S, Del Greco A, This D, Baum M, Ceccarelli S (2008) Quantitative trait loci associated with adaptation to Mediterranean dryland conditions in barley. Theor Appl Genet 117(5):653–669

von Korff M, Léon J, Pillen K (2010) Detection of epistatic interactions between exotic alleles introgressed from wild barley (*H. vulgare* ssp. *spontaneum*). Theor Appl Genet 121(8):1455–1464

Waddington SR, Cartwright PM, Wall PC (1983) A quantitative scale of spike initial and pistil development in barley and wheat. Ann Bot 51:119–130

Wang G, Schmalenbach I, von Korff M, Léon J, Kilian B, Rode J, Pillen K (2010) Association of barley photoperiod and vernalization genes with QTLs for flowering time and agronomic traits in a DH-population and a set of wild barley introgression lines. Theor Appl Genet 120 (8):1559–1574

Weltzien E (1988) Evaluation of barley (*Hordeum vulgare* L) landrace populations originating from different growing regions in the near East. Plant Breed 101:95–106

Weltzien E (1989) Differentiation among barley landrace populations from the near East. Euphytica 43:29–39

Whitechurch EM, Slafer GA, Miralles DJ (2007) Variability in the duration of stem elongation in wheat genotypes and sensitivity to photoperiod and vernalization. J Agron Crop Sci 193 (2):131–137

Wilhelm EP, Turner AS, Laurie DA (2009) Photoperiod insensitive *Ppd-A1a* mutations in tetraploid wheat (*Triticum durum Desf.*). Theor Appl Genet 118:285–294

Worland T, Snape JW (2001) Genetic basis of worldwide wheat varietal improvement. In: Bonjean AP, Angus WJ (eds) The world wheat book: a history of wheat breeding. Lavoisier Publishing, Paris, pp 59–100

Xue W, Xing Y, Weng X, Zhao Y, Tang W, Wang L, Zhou H, Yu S, Xu C, Li X, Zhang Q (2008) Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. Nat Genet 6:761–767

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene *VRN1*. Proc Natl Acad Sci U S A 100:6263–6268

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1644

Yan L, Fu D, Li C, Blechl A, Tranquilli M, Bonafede A, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. Proc Natl Acad Sci U S A 103:19581–19586

Yasuda S, Hayashi J, Moriya I (1993) Genetic constitution for spring growth habit and some other characters in barley cultivars in the Mediterranean coastal regions. Euphytica 70:77–83

Yin X (2005) Crop system dynamics: an ecophysiological simulation model for genotype-by-environment interactions. Wageningen Academic, Wageningen

Zakhrabekova S, Gough SP, Braumann I, Müller AH, Lundqvist J, Ahmann K, Dockter C, Matyszczak I, Kurowska M, Druka A, Waugh R, Graner A, Stein N, Steuernagel B, Lundqvist U, Hansson M (2012) Induced mutations in circadian clock regulator Mat-a facilitated short-season adaptation and range extension in cultivated barley. Proc Natl Acad Sci U S A 109:4326–4331

Chapter 6
Improvement of Mineral Nutrition: A Source and Sink for Candidate Genes

Benjamin D. Gruber and Nicolaus von Wirén

6.1 Introduction

Barley (*Hordeum vulgare*) is grown in many areas throughout the world, each differing greatly in their soil characteristics. Over the 10 years from 2000 to 2009, the major producers of barley were the Russian Federation, Germany, Canada, France, Ukraine, Turkey, Australia, Spain, the United Kingdom and the United States of America (FAOSTAT 2011). Soil types among and within these countries are widely varied, and the pH ranges from alkaline through to acidic (FAO Land Resources 2011). Such a wide variety of soil types present to plants large differences in the availability of the mineral elements required for growth. Additionally, some of these soils contain elements that are present at concentrations toxic to plants, and indeed mineral deficiency and toxicity occur concurrently within single soil environments. In response to this, strategies that overcome some of these problems have developed throughout the evolution and breeding of barley. However, there are instances where barley is weak at coping with specific elemental conditions relative to other crop plants. As such there are two biotechnological focus points relating to barley. The first relates to the mechanisms that barley is good at and that can be taken and coadapted for other purposes or for use in other species. The second relates to the improvement of barley through the introduction of mechanisms taken from other species. In this review we will discuss some examples from both focus points with an emphasis on three desires within mineral plant nutrition, namely, increasing tolerance to nutrient deficiency or elemental toxicity, increasing nutrient use efficiency and increasing the loading of nutrients into the grain.

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6.2 Soil Factors Affecting Nutrient Availability

The availability of nutrients is heavily dependent upon many soil factors and often involves complex chemical interactions. Different soils contain varying concentrations of elements due to soil genesis or to more recent nutrient management regimes applied by land managers. Aspects of soil chemistry then affect the degree to which these soil nutrients are available to plants. The pH and the content of water, organic matter and salt within the soil are among the most influential factors. Soil pH can have a drastic influence upon the availability of nutrients for uptake by barley. Alkaline soils with a pH above 7 are often characterised by the reduced availability of iron (Fe), zinc (Zn), manganese (Mn) and phosphorus (P). Additionally, acid soils with a pH below 5.5 also affect many areas where barley is grown. Within these soils the availability of elements such as P are reduced, while the available concentrations of elements such as aluminium (Al) and Mn increase to levels that inhibit root growth and negatively affect overall plant growth.

A reduction in the soil water content first decreases those nutrients that are transported to the root surface by mass flow, such as nitrate, sulphate or calcium. Thus, drought stress during the vegetative growth phase, when nutrient demand is high, may result in symptoms of nitrogen deficiency before wilting occurs. Even lower water potentials will decrease the transport of diffusion-driven nutrients such as potassium and phosphate. Drought-prone sites are often associated with salinity, especially when salt is transported from groundwater tables to the soil surface by evaporation (Rengasamy 2006). Elevated levels of sodium chloride decrease water availability to roots and affect nutrient uptake and ion balance within the plant.

6.3 Aluminium Tolerance

Acid soils affect 30 % of the world's ice-free land including many areas where barley is grown (Uexküll and Mutert 1995). The availability of toxic Al^{3+} cations increases, thereby negatively affecting root growth by inhibiting cell division and expansion at the root apex (Kochian et al. 2005). Plants cope with toxic levels of Al by either excluding it from the plant [defined as Al resistance by Delhaize et al. (2007)] or by storing it in less toxic forms or less susceptible locations (defined as Al tolerance). Aluminium resistance in many cereals is predominantly dependent upon the exclusion of Al through the release of organic acids into the rhizosphere (Kochian et al. 2005). The organic acid anions chelate the phytotoxic Al^{3+} cations reducing their toxicity and preventing their uptake by the plant. Malate, citrate and oxalate are the three predominant organic anions that are released by cereal species. Barley releases citrate from roots; however, the quantity it releases is relatively low resulting in poor Al resistance (Zhao et al. 2003).

In wheat the release of malate is governed by the action of the organic anion channel TaALMT1 (*Triticum aestivum* aluminium-activated malate transporter)

that is activated in the presence of external Al (Sasaki et al. 2004). There are considerable differences in the level of malate efflux (and therefore Al resistance) between wheat genotypes. It was established that Al-resistant genotypes possess multiple promoter elements that drive *TaALMT1* expression, indicating that the modulation of *TaALMT1* expression alone is enough to enhance Al resistance in wheat. This concept then lends itself to the overexpression of *ALMT* genes in heterologous systems to increase their Al resistance.

When the *TaALMT1* gene was constitutively overexpressed in barley under the control of the ubiquitin promoter, the malate efflux from root apices increased relative to control plants when exposed to Al. The increased efflux of malate correlated with an increase in Al resistance in nutrient solution containing Al as well as in an acidic soil, where barley root length was no longer repressed by Al and transgenic plants, produced greater grain yields (Delhaize et al. 2004, 2009). Additionally, transgenic plants were more efficient at utilising P. The authors suggest that this might be due to the malate release increasing the mobilisation of P from the soil or the malate providing additional protection to the P uptake mechanisms against Al (Delhaize et al. 2009).

Interestingly, ALMT proteins are found across many plant species with barley possessing at least two members; however, HvALMT1 (the most similar of these to TaALMT1) is not responsible for the small degree of Al resistance seen in barley (Delhaize et al. 2007; Gruber et al. 2010). Instead a MATE protein is responsible for the exudation of citrate in response to Al (Furukawa et al. 2007; Wang et al. 2007). HvALMT1 exhibits one major difference to TaALMT1 in that the efflux of organic acid anions through the channel is constitutive and therefore not activated by Al (Gruber et al. 2010). It was proposed that the protein is involved in the transport of organic acids in the guard cells of stomata and adjacent to root tips, where *HvALMT1* expression was localised. Constitutive overexpression of *HvALMT1* produced stunted plants (Gruber et al. 2011). Perhaps this was because the loss of organic compounds via root exudation was too great or because the ectopic function of the protein was detrimental for plant growth. Nevertheless, this protein is a candidate for root-specific overexpression as it effluxes organic acids in the absence of Al activation and may therefore be useful for facilitating P solubilisation even in soils lacking Al.

While traditional mechanisms of Al tolerance are good targets for biotechnology, it is also possible to modify other processes to contribute to this trait. For example, overexpression of a thioredoxin gene from *Phalaris coerulescens* slightly enhances the root growth of barley germinated in conditions of moderate Al toxicity (Li et al. 2010). Thioredoxin proteins are critical for the control of the redox state of proteins and have been shown to be involved in the plant defence response to oxidative stress (Vieira Dos Santos and Rey 2006) such as those that may occur under conditions of Al toxicity (Panda and Matsumoto 2007). Although the benefits revealed in the study of Li et al. (2010) are fairly small, such approaches are worthy of further investigation.

6.4 Iron Acquisition

Iron acquisition in graminaceous plant species relies upon the so-called strategy II mechanism, which is specific to grasses. This mechanism relies upon the Fe deficiency-induced exudation of phytosiderophores (PS) that chelate Fe in a ferric complex that is then taken up by the plant (Romheld 1987). Among the grasses barley is one of the most tolerant to the development of chlorosis caused by Fe deficiency. This is because barley releases large amounts of PS in response to Fe deficiency as a very effective method to increase Fe solubilisation and subsequent uptake (Marschner et al. 1987). These PS include mugineic acid (MA) and 2-′-deoxymugineic acid (DMA), which are hexadentate chelators with high affinity to ferric Fe. Such efficient Fe acquisition machinery makes barley a useful source of components for transfer to other species less able to acquire poorly soluble forms of Fe.

The pathway for the biosynthesis of PS in barley has been determined hence clearing the way for their exploitation in biotechnological approaches (Bashir et al. 2006; Higuchi et al. 1999; Nakanishi et al. 2000; Takahashi et al. 1999). The release of the PS DMA is governed by the activity of the HvTOM1 transporter that is a member of the major facilitator superfamily (Nozoye et al. 2011). *HvTOM1* is expressed under Fe deficiency throughout many cell layers of the root including the epidermis, thereby releasing PS that bind Fe in the apoplast and/or rhizosphere. The $Fe^{(III)}$-PS (e.g. MA or DMA) complex is then taken up through HvYS1, a member of the oligopeptide transporter (OPT) family. The HvYS1 transporter is localised on the plasma membrane of root epidermal cells, induced under Fe deficiency and appears specific for the uptake of $Fe^{(III)}$-PS but not other metal-phytosiderophore complexes (Murata et al. 2006, 2008). Overexpression of *HvTOM1* or *TOM1* from rice (*Oryza sativa*) resulted in less chlorosis in transgenic rice plants when grown under Fe deficiency (Nozoye et al. 2011).

NAAT (nicotianamine aminotransferase) undertakes the first step in the conversion of nicotianamine (NA) to the precursor of DMA (Takahashi et al. 1999). Two *NAAT* genes from barley were expressed in rice under the transcriptional control of their native barley promoters (Takahashi et al. 2001). This generally replicated the Fe-deficient expression pattern in roots that had been previously observed in barley. Transgenic rice grown indoors on an alkaline substrate exhibited fewer symptoms of Fe deficiency and grew taller than control plants and produced greater shoot biomass and grain yield. While the growth was substantially increased, transgenic plants released only 1.8 times more DMA than non-transgenic plants under Fe deficiency. The authors proposed that this may have been due to a shortage of the NA precursor.

Transgenic rice plants overexpressing *HvNAS1* (NA synthase), *HvIDS3* (converts DMA to MA) and *HvNAS1* combined with the two *HvNAAT* genes were grown on a calcareous field (Suzuki et al. 2008). Transgenic plants showed fewer signs of Fe deficiency-induced chlorosis and grew taller during the growth phase; however, no differences in height were observed at harvesting. Grain yields tended

to be greater for the *HvNAS1* transgenic plants; however, the difference was marginal for the other transgenic plants. As expected, the growth benefits arising from the overexpression of these three genes were observed only on calcareous soil as they were absent when lines from the same group were grown in a noncalcareous Andosol soil (Masuda et al. 2008).

6.5 Mineral Translocation and Biofortification of Grains

One of the major challenges for crop improvement is the production of food products with increased nutritional benefits to the consumer. Since Fe deficiency affects 60 % and Zn deficiency 30 % of the world's population (White and Broadley 2009), the development of cereal crops with increased Fe and Zn content in the grains is one of the major breeding goals. The review of White and Broadley (2009) found in a literature search that there was a 3.95 times difference reported between the barley genotype with the highest and the genotype with the lowest concentration of Fe in the grain. While less than rice, the variation among genotypes of barley was similar to wheat. Many families of proteins are prime candidates for the modification of biofortification in barley. This is because the process of biofortification requires the coordination of a number of steps along the nutrient transport pathway, first uptake from the soil, then transport into the xylem, possible remobilisation from the leaf to the grain and then finally loading into the grain itself (Borg et al. 2009). The list of potential candidates is therefore too large to discuss in detail here; however, readers are directed to the review of Waters and Sankaran (2011) that describes genes with potential roles in biofortification, as part of either their native function or a biotechnological approach.

The acquisition of nutrients such as Fe from the soil is just one component of the complement of strategies that plants require to maintain elemental homeostasis and to increase the efficiency of nutrient use. Subsequently, nutrients must be translocated from the roots to leaves. In the generative growth phase, many nutrients are remobilised from leaves to be delivered to sink organs including the grain, especially as the plant progresses into senescence. As with the uptake of Fe from the soil, NA plays a role here as Fe-NA complexes appear to be translocated between tissues (Takahashi et al. 2003). Additionally, the genes encoding for enzymes of the PS synthesis pathway are expressed in the phloem of leaves (Bashir et al. 2006), as are transporters of metal-PS or metal-NA complexes in rice (e.g. TOM1, OsYSL2, OsYSL15, OsYSL18; Aoyama et al. 2009; Inoue et al. 2009; Koike et al. 2004; Nozoye et al. 2011). The overexpression of a number of the synthesis and transport components from barley, both constitutively and using tissue-specific promoters, has resulted in the increased concentration of mineral elements such as Fe, Cu and Zn in rice grains (Masuda et al. 2008, 2009; Nozoye et al. 2011; Suzuki et al. 2008).

To date we are aware of just one protein that has been overexpressed in barley that results in an increase in the mineral nutrient concentration within the barley

grain. AtZIP1 is a member of the ZRT/IRT-related protein (ZIP) family reviewed by Guerinot (2000). These membrane transport proteins typically contain metal-binding domains, and the majority of the proteins in the family have roles in the transport of metals such as Fe and Zn. *AtZIP1* is mainly expressed in the roots of *Arabidopsis thaliana* during Zn limitation and functions as a Zn transporter (Grotz et al. 1998). Overexpression of *AtZIP1* in barley conferred an increase in the short-term uptake of Zn into both the shoots and roots of plants grown under Zn-deficient conditions (Ramesh et al. 2004). While Zn content was not increased over the long term, the growth of transgenic plants was increased relative to wild-type plants. Additionally, the loading of Zn and Fe into the grain was increased by up to twofold in transgenic plants.

6.6 Boron

In most soils boron (B) availability is high since most B occurs as boric acid which is a neutral molecule undergoing little interaction with the soil matrix. In this form, B is readily absorbed by roots, partially diffusing directly through the plasma membrane (Dordas and Brown 2000). In B-deficient soils, roots increase B uptake by inducing the expression of NIP-type transporters to increase B influx into roots and of BOR1-type transporters that mediate xylem loading (Takano et al. 2002, 2006). The employment of these transporter sequences in biotechnological approaches to improve B acquisition would be supported by the B-sensitive post-transcriptional regulation that leads to RNA or protein degradation after B resupply thus preventing B toxicity in overexpression lines (Takano et al. 2005; Tanaka et al. 2011).

In soils containing high levels of B, large amounts can be transported via mass flow to the roots and then accumulate to levels that result in toxicity. Such toxicity is characterised by chlorosis and necrosis developing first at the tip of leaf blades and progressing along the leaf as toxicity develops (Schnurbusch et al. 2010a). Mechanisms of tolerance rely primarily on excluding B from tissues by increasing the export of B from the plant, typically from the root (Hayes and Reid 2004) or from the leaf tip by secretion via guttation (Oertli 1962). Genotypes tolerant of B therefore contain lower leaf concentrations of B than their sensitive counterparts. By molecular cloning of a B tolerance locus in barley, a *BOR1* homologue was identified, named *BOT1* or *HvBOR2*, which is responsible for the high B tolerance of the cultivar Sahara 3771 relative to the cultivar Clipper (Reid and Fitzpatrick 2009; Sutton et al. 2007). In addition to the much greater transcript levels of *BOT1* found in Sahara 3771, the protein from Sahara 3771 conferred greater tolerance to B toxicity than the protein from Clipper when overexpressed in yeast (Sutton et al. 2007). Two amino acid residues were critical in increasing the B transport capacity hence explaining the difference in B transport between the proteins from the two cultivars. The *BOT1* gene is expressed in the meristem and elongation zone of roots and in the mesophyll of mature leaf blades with expression strongest at the

leaf tip. BOT1 appears to function in the removal of B from roots by transporting it back into the surrounding medium (Reid 2007; Sutton et al. 2007) and in transporting B from the symplast to the apoplast in leaves (Reid and Fitzpatrick 2009).

An additional B tolerance locus in barley harbours the aquaporin-related gene *HvNIP2;1* (Schnurbusch et al. 2010b). HvNIP2;1 was shown to be capable of importing B in yeast. Gene expression was detected only in the roots of barley and was detected at a greater level in the sensitive genotype Clipper. The level of B accumulation was 25 % less in the leaves of Sahara 3771 than Clipper. It was proposed that the reduced expression of *HvNIP2;1* increases the B tolerance of Sahara 3771 as the protein normally facilitates the uptake of B into the plant, and this is reduced in the tolerant genotype (Schnurbusch et al. 2010b).

The *BOT1* gene from the tolerant genotype Sahara is a potential source for the biotechnological improvement of not just barley but other plant species as well. Indeed, it was found that conventional breeding often results in the carryover of negative traits associated with or in close linkage with the *BOT1* locus (Sutton et al. 2007). Although recent breeding approaches appear to have partially separated the closely linked traits, it may also be useful to utilise genetic transformation to introduce B tolerance while hopefully reducing the appearance of the negative traits associated with the locus (Reid 2010; Schnurbusch et al. 2010a). It might also be worth testing if increasing the expression of *BOT1* to even greater levels would provide a further additive increase in B tolerance.

6.7 Salt Tolerance

Cereals possess multiple mechanisms of salinity tolerance that have been comprehensively reviewed by Munns et al. (2006) and Munns and Tester (2008); hence, we consider only a couple of points that may be of greatest relevance for this chapter. Of the cereals, barley is the most tolerant to salinity and relies mainly upon the tissues being tolerant to greater concentrations of the Na^+ arising from the salt (Munns and Tester 2008). High concentrations of Na^+ in the cytoplasm inhibit enzymes and compete with K^+ for the binding sites of proteins, so typically Na^+ is sequestered into the vacuole to keep the cytoplasmic concentrations low (James et al. 2006). In barley it was also found that Na^+ accumulates more in the vacuoles of mesophyll cells than epidermal cells, where a lower Na^+ concentration and higher K^+:Na^+ were maintained in the cytoplasm (James et al. 2006).

The exclusion of salt from the plant is the salt tolerance mechanism that the majority of plants utilise. To be effective plants must exclude at least approximately 98 % of the salt in the soil solution; otherwise salt will start to accumulate (Munns et al. 2006). Barley excludes only about 94 % of the salt in the soil, hence accumulating Na^+ in shoots and thereby necessitating mechanisms of tissue tolerance (Munns 2005). Na^+ tolerance can also be achieved by preventing Na^+ from entering the xylem and thereby the transpiration stream to the leaves. Such an

alternative mechanism of salt tolerance is dominant in wheat and relies, at least partly, on the selectivity of transporters for K^+ over Na^+ (Munns et al. 2006). Barley is relatively weak at excluding salt, and the exclusion mechanisms have a weaker K^+:Na^+ selectivity (Gorham et al. 1990). The retrieval of Na^+ from the xylem is another key component of Na^+ tolerance. In rice, members of the high-affinity K^+ transporter (HKT) family have been implicated in Na^+ accumulation in xylem parenchyma cells in roots, thus limiting the net translocation of Na^+ to the leaves (Ren et al. 2005). Across plants the HKT family consists of two subfamilies, the first of which is relatively selective for Na^+ and the second of which acts to transport both Na^+ and K^+. Multiple *HKT* genes from both subfamilies have been identified in barley; however, the roles of these are yet to be fully elucidated (Huang et al. 2008).

Overexpression of *HvHKT2;1*, the only member of HKT subfamily II in barley, resulted in an increase in the salt tolerance of transgenic barley plants (Mian et al. 2011). The constitutive expression conferred an increase in salt translocation to the shoot via the xylem such that the Na^+ concentration in leaves increased in transgenic plants. The authors proposed that the stimulated Na^+ translocation "reinforced the salt includer phenotype of barley". To elaborate on this unexpected observation, they suggest that the extra Na^+ accumulation reinforces other components involved in salt tolerance such as the compartmentalisation of Na^+. The rice homologue of *HvHKT2;1* (*OsHKT2;1*) is expressed in the cortex of roots and in leaves and is normally downregulated in response to salt exposure (Horie et al. 2007; Kader et al. 2006). This would contradict the result presented here, although perhaps differences arise from the fact that rice is salt sensitive and poorly able to tolerate salt in the leaves (Munns and Tester 2008). The transgenic plants and the hypothesis developed to explain their phenotype would indeed mirror the native salt tolerance strategy of barley. However, from a biotechnological perspective it would be more elegant to use a targeted approach whereby Na^+ uptake is decreased or Na^+ export is increased without an associated net increase in Na^+ uptake. For instance, this may be brought about by the overexpression of genes of *HKT* subfamily I or SOS1-type Na^+/H^+ antiporters (Shi et al. 2003). Alternatively, Na^+ translocation to the shoot can be suppressed by targeted overexpression of HKT1 homologues in the root stele, as successfully shown in Arabidopsis (Møller et al. 2009). Such a genetic stacking of salt exclusion mechanisms might then complement the endogenous tissue tolerance that already exists in barley.

Outlook and Conclusion

The above-mentioned examples show how barley can serve as a source or a sink for the biotechnological modification of traits relating to the uptake, translocation and tolerance of mineral elements. Barley has proven an excellent source of useful genes for processes for which it is well adapted, for example, the efficient acquisition and translocation of Fe that helps under alkaline growth conditions. On the other hand, barley is poorly adapted to

(continued)

acidic soils and is therefore more of a sink, i.e. a recipient of genes related to processes that maintain growth in these soils (e.g. aluminium resistance).

Further improvements in the mineral nutrition of barley are likely to come from the use of gene stacking to introduce multiple traits. Although efficient Fe uptake and salt tolerance strategies already exist in barley, genotypes that are most efficient for individual components (i.e. salt exclusion, tissue tolerance, Fe uptake) could be utilised as a source of genes to develop cultivars that are best optimised for combinations of such traits. The existing strategies could be further complemented by pyramiding genes sourced from other species, such as those for Al resistance from wheat, for example, into barley for an even wider spectrum of improved nutritional traits.

Current biotechnological strategies have mainly centred on physiological means of enhancing mineral nutrition. Indeed such a focus will most likely bring substantial improvements in the use of barley across a wider range of agricultural environments. However, morphological traits in barley have been relatively poorly explored in a biotechnological context, even though they may also bring improvements in mineral nutrition. The use of traits relating to root morphology, for example, the length, number and distribution of different orders of roots and root hairs, is also likely to enhance mineral nutrition. Such benefits are most likely to arise from aspects of nutrient uptake due to better soil exploration and foraging and more efficient allocation of resources for nutrient capture. Work on identifying such traits and their genetic basis is indeed underway in barley, and it will be interesting to track such developments and explore to what extent physiological and morphological traits will add up to further improve mineral nutrition.

References

Aoyama T, Kobayashi T, Takahashi M, Nagasaka S, Usuda K, Kakei Y, Ishimaru Y, Nakanishi H, Mori S, Nishizawa N (2009) OsYSL18 is a rice iron(III)–deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints. Plant Mol Biol 70:681–692

Bashir K, Inoue H, Nagasaka S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2006) Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. J Biol Chem 281:32395–32402

Borg S, Brinch-Pedersen H, Tauris B, Holm PB (2009) Iron transport, deposition and bioavailability in the wheat and barley grain. Plant Soil 325:15–24

Delhaize E, Ryan PR, Hebb DM, Yamamoto Y, Sasaki T, Matsumoto H (2004) Engineering high-level aluminum tolerance in barley with the *ALMT1* gene. Proc Natl Acad Sci U S A 101:15249–15254

Delhaize E, Gruber BD, Ryan PR (2007) The roles of organic anion permeases in aluminium resistance and mineral nutrition. FEBS Lett 581:2255–2262

Delhaize E, Taylor P, Hocking PJ, Simpson RJ, Ryan PR, Richardson AE (2009) Transgenic barley (*Hordeum vulgare* L.) expressing the wheat aluminium resistance gene (*TaALMT1*)

shows enhanced phosphorus nutrition and grain production when grown on an acid soil. Plant Biotechnol J 7:391–400
Dordas C, Brown PH (2000) Permeability of boric acid across lipid bilayers and factors affecting it. J Membr Biol 175:95–105
FAOSTAT (2011) Food and agriculture organization of the United Nations. http://faostat.fao.org. Cited 12 Sep 2011
Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF (2007) An aluminum-activated citrate transporter in barley. Plant Cell Physiol 48:1081–1091
Gorham J, Bristol A, Young EM, Jonesh RGW, Kashour G (1990) Salt tolerance in the Triticeae: K/Na discrimination in barley. J Exp Bot 41:1095–1101
Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. Proc Natl Acad Sci U S A 95:7220–7224
Gruber BD, Ryan PR, Richardson AE, Tyerman SD, Ramesh S, Hebb DM, Howitt SM, Delhaize E (2010) HvALMT1 from barley is involved in the transport of organic anions. J Exp Bot 61:1455–1467
Gruber BD, Delhaize E, Richardson AE, Roessner U, James RA, Howitt SM, Ryan PR (2011) Characterisation of *HvALMT1* function in transgenic barley plants. Funct Plant Biol 38:163–175
Guerinot ML (2000) The ZIP family of metal transporters. BBA Biomembr 1465:190–198
Hayes JE, Reid RJ (2004) Boron tolerance in barley is mediated by efflux of boron from the roots. Plant Physiol 136:3376–3382
Higuchi K, Suzuki K, Nakanishi H, Yamaguchi H, Nishizawa N-K, Mori S (1999) Cloning of nicotianamine synthase genes, novel genes involved in the biosynthesis of phytosiderophores. Plant Physiol 119:471–480
Horie T, Costa A, Kim TH, Han MJ, Horie R, Leung H-Y, Miyao A, Hirochika H, An G, Schroeder JI (2007) Rice OsHKT2;1 transporter mediates large Na^+ influx component into K^+-starved roots for growth. EMBO J 26:3003–3014
Huang S, Spielmeyer W, Lagudah ES, Munns R (2008) Comparative mapping of *HKT* genes in wheat, barley, and rice, key determinants of Na^+ transport, and salt tolerance. J Exp Bot 59:927–937
Inoue H, Kobayashi T, Nozoye T, Takahashi M, Kakei Y, Suzuki K, Nakazono M, Nakanishi H, Mori S, Nishizawa NK (2009) Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. J Biol Chem 284:3470–3479
James RA, Munns R, Von Caemmerer S, Trejo C, Miller C, Condon T (2006) Photosynthetic capacity is related to the cellular and subcellular partitioning of Na^+, K^+ and Cl^- in salt-affected barley and durum wheat. Plant Cell Environ 29:2185–2197
Kader MA, Seidel T, Golldack D, Lindberg S (2006) Expressions of OsHKT1, OsHKT2, and OsVHA are differentially regulated under NaCl stress in salt-sensitive and salt-tolerant rice (*Oryza sativa* L.) cultivars. J Exp Bot 57:4257–4268
Kochian LV, Pineros MA, Hoekenga OA (2005) The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. Plant Soil 274:175–195
Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant J 39:415–424
FAO Land Resources (2011) Food and agriculture organization of the United Nations. http://www.fao.org/nr/land/lr-home/en/. Cited 13 Sep 2011
Li QY, Niu HB, Yin J, Shao HB, Niu JS, Ren JP, Li YC, Wang X (2010) Transgenic barley with overexpressed *PTrx* increases aluminum resistance in roots during germination. J Zhejiang Univ Sci B 11:862–870
Marschner H, Romheld V, Kissel M (1987) Localization of phytosiderophore release and of iron uptake along intact barley roots. Physiol Plantarum 71:157–162

Masuda H, Suzuki M, Morikawa KC, Kobayashi T, Nakanishi H, Takahashi M, Saigusa M, Mori S, Nishizawa NK (2008) Increase in iron and zinc concentrations in rice grains via the introduction of barley genes involved in phytosiderophore synthesis. Rice 1:100–108

Masuda H, Usuda K, Kobayashi T, Ishimaru Y, Kakei Y, Takahashi M, Higuchi K, Nakanishi H, Mori S, Nishizawa NK (2009) Overexpression of the barley nicotianamine synthase gene *HvNAS1* increases iron and zinc concentrations in rice grains. Rice 2:155–166

Mian A, Oomen R, Isayenkov S, Sentenac H, Maathuis FJM, Very AA (2011) Over-expression of an Na^+- and K^+-permeable HKT transporter in barley improves salt tolerance. Plant J 68:468–479

Møller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, Haseloff J, Tester M (2009) Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type–specific alteration of Na^+ transport in *Arabidopsis*. Plant Cell 21:2163–2178

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167:645–663

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Munns R, James RA, Läuchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot 57:1025–1043

Murata Y, Ma JF, Yamaji N, Ueno D, Nomoto K, Iwashita T (2006) A specific transporter for iron (III)–phytosiderophore in barley roots. Plant J 46:563–572

Murata Y, Harada E, Sugase K, Namba K, Horikawa M, Ma JF, Yamaji N, Ueno D, Nomoto K, Iwashita T, Kusumoto S (2008) Specific transporter for iron(III)-phytosiderophore complex involved in iron uptake by barley roots. Pure Appl Chem 80:2689–2697

Nakanishi H, Yamaguchi H, Sasakuma T, Nishizawa NK, Mori S (2000) Two dioxygenase genes, Ids3 and Ids2, from *Hordeum vulgare* are involved in the biosynthesis of mugineic acid family phytosiderophores. Plant Mol Biol 44:199–207

Nozoye T, Nagasaka S, Kobayashi T, Takahashi M, Sato Y, Sato Y, Uozumi N, Nakanishi H, Nishizawa NK (2011) Phytosiderophore efflux transporters are crucial for iron acquisition in graminaceous plants. J Biol Chem 286:5446–5454

Oertli JJ (1962) Loss of boron from plants through guttation. Soil Sci 94:214–219

Panda SK, Matsumoto H (2007) Molecular physiology of aluminum toxicity and tolerance in plants. Bot Rev 73:326–347

Ramesh SA, Choimes S, Schachtman DP (2004) Over-expression of an *Arabidopsis* zinc transporter in *Hordeum vulgare* increases short-term zinc uptake after zinc deprivation and seed zinc content. Plant Mol Biol 54:373–385

Reid R (2007) Identification of boron transporter genes likely to be responsible for tolerance to boron toxicity in wheat and barley. Plant Cell Physiol 48:1673–1678

Reid R (2010) Can we really increase yields by making crop plants tolerant to boron toxicity? Plant Sci 178:9–11

Reid R, Fitzpatrick K (2009) Influence of leaf tolerance mechanisms and rain on boron toxicity in barley and wheat. Plant Physiol 151:413–420

Ren Z-H, Gao J-P, Li L-G, Cai X-L, Huang W, Chao D-Y, Zhu M-Z, Wang Z-Y, Luan S, Lin H-X (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37:1141–1146

Rengasamy P (2006) World salinization with emphasis on Australia. J Exp Bot 57:1017–1023

Romheld V (1987) Different strategies for iron acquisition in higher plants. Physiol Plantarum 70:231–234

Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminium-activated malate transporter. Plant J 37:645–653

Schnurbusch T, Hayes J, Sutton T (2010a) Boron toxicity tolerance in wheat and barley: Australian perspectives. Breed Sci 60:297–304

Schnurbusch T, Hayes J, Hrmova M, Baumann U, Ramesh SA, Tyerman SD, Langridge P, Sutton T (2010b) Boron toxicity tolerance in barley through reduced expression of the multifunctional aquaporin HvNIP2;1. Plant Physiol 153:1706–1715

Shi H, Lee B-H, Wu S-J, Zhu J-K (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nat Biotechnol 21:81–85

Sutton T, Baumann U, Hayes J, Collins NC, Shi BJ, Schnurbusch T, Hay A, Mayo G, Pallotta M, Tester M, Langridge P (2007) Boron-toxicity tolerance in barley arising from efflux transporter amplification. Science 318:1446–1449

Suzuki M, Morikawa KC, Nakanishi H, Takahashi M, Saigusa M, Mori S, Nishizawa NK (2008) Transgenic rice lines that include barley genes have increased tolerance to low iron availability in a calcareous paddy soil. Soil Sci Plant Nutr 54:77–85

Takahashi M, Yamaguchi H, Nakanishi H, Shioiri T, Nishizawa NK, Mori S (1999) Cloning two genes for nicotianamine aminotransferase, a critical enzyme in iron acquisition (strategy II) in graminaceous plants. Plant Physiol 121:947–956

Takahashi M, Nakanishi H, Kawasaki S, Nishizawa NK, Mori S (2001) Enhanced tolerance of rice to low iron availability in alkaline soils using barley nicotianamine aminotransferase genes. Nat Biotechnol 19:466–469

Takahashi M, Terada Y, Nakai I, Nakanishi H, Yoshimura E, Mori S, Nishizawa NK (2003) Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. Plant Cell 15:1263–1280

Takano J, Noguchi K, Yasumori M, Kobayashi M, Gajdos Z, Miwa K, Hayashi H, Yoneyama T, Fujiwara T (2002) Arabidopsis boron transporter for xylem loading. Nature 420:337–340

Takano J, Miwa K, Yuan L, von Wirén N, Fujiwara T (2005) Endocytosis and degradation of BOR1, a boron transporter of *Arabidopsis thaliana*, regulated by boron availability. Proc Natl Acad Sci U S A 102:12276–12281

Takano J, Wada M, Ludewig U, Schaaf G, von Wirén N, Fujiwara T (2006) The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 18:1498–1509

Tanaka M, Takano J, Chiba Y, Lombardo F, Ogasawara Y, Onouchi H, Naito S, Fujiwara T (2011) Boron-dependent degradation of NIP5;1 mRNA for acclimation to excess boron conditions in Arabidopsis. Plant Cell 23:3547–3559

Uexküll HR, Mutert E (1995) Global extent, development and economic impact of acid soils. Plant Soil 171:1–15

Vieira Dos Santos C, Rey P (2006) Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci 11:329–334

Wang JP, Raman H, Zhou MX, Ryan PR, Delhaize E, Hebb DM, Coombes N, Mendham N (2007) High-resolution mapping of the *Alp* locus and identification of a candidate gene *HvMATE* controlling aluminium tolerance in barley (*Hordeum vulgare* L.). Theor Appl Genet 115:265–276

Waters BM, Sankaran RP (2011) Moving micronutrients from the soil to the seeds: genes and physiological processes from a biofortification perspective. Plant Sci 180:562–574

White PJ, Broadley MR (2009) Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol 182:49–84

Zhao Z, Ma JF, Sato K, Takeda K (2003) Differential Al resistance and citrate secretion in barley (*Hordeum vulgare* L.). Planta 217:794–800

Chapter 7
Photosynthesis and Leaf Senescence as Determinants of Plant Productivity

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7.1 Introduction: Regulation of Photosynthesis and Crop Plant Productivity

The efficiency of the conversion from light to metabolic energy in photosynthesis sets a ceiling for maximal crop yields (Zhu et al. 2010). Therefore, any attempt to enhance the productivity of crop plants beyond current levels will have to incorporate improvements in photosynthetic efficiency, as well as extending the duration of assimilate production. The development of novel strategies for improvements in crop performance with direct regard to enhancing photosynthetic functions will require an increase in maximal photosynthetic capacity and in photosynthetic efficiency per leaf area (1), an increase in the light interception by the crop canopy (2) and an extension of the period when maximal light absorbance is achieved, e.g. by modulating the early leaf emergence and the senescence process of the crop (3) (Parry et al. 2011). Other options linked to photosynthesis such as an increase in the capacity for sucrose synthesis and/or export properties might be used to increase crop performance by manipulation of source–sink relationships and harvest index. Indeed, the increases in cereal grain yields that have occurred since the 1950s, particularly in wheat, were largely obtained by increases in harvest index, i.e. the partitioning of photosynthates to the grain (Austin et al. 1980). However, since the

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early 1980s, increases in plant biomass linked to the production of photosynthates have also been an important component of crop yield increases (Reynolds et al. 2005). Such increases have undoubtedly incorporated improvements in radiation use efficiency, which has probably been achieved by better light interception by the canopy due to changes in plant architecture, such as the use of cultivars having erect leaves (Innes and Blackwell 1983).

Increasing radiation use efficiency in order to increase crop yields is not a trivial undertaking because the whole-plant physiology, particularly source–sink relationships, has to adjust to the increased efficiency of the photosynthetic output in order to gain maximal benefit in terms of carbon gain. Restrictions on the source–sink interaction, particularly in the transport of photosynthates, currently limit the exploitation of enhanced photosynthetic capacities in modern cereal crops (Reynolds et al. 2005; Bingham et al. 2007). Hence, the source–sink interaction has also to be improved in order to exploit fully any potential improvements in radiation use efficiency.

The molecular mechanisms that contribute to stay-green phenotypes also have considerable potential for exploitation in breeding strategies aimed at enhancing productivity by prolonging leaf lifespan. Yield potential can be increased through stay-green mechanisms that maintain green leaf area for longer time. Stay-green mechanisms allow leaves to retain chlorophyll and other pigments in photosynthetic tissues at the end of the crop cycle, which may translate to a higher biomass accumulation and grain yields particularly under stress conditions. Stay-green phenotypes can have a positive role in increasing the amount of light used effectively in photosynthesis to drive biomass production, but they are often associated with other traits, such as a slow leaf growth rate. Finally, exposure to biotic stresses such as pathogen attack and abiotic stresses such as extremes of light and temperature, nutrient deficiencies and drought has a profound effect on the regulation and function of the photosynthetic apparatus and on the onset of the senescence programme. Therefore the complexity and fine-tuning of the regulatory interplay between the photosynthetic apparatus and leaf senescence in relation to its importance as a major determinant of crop productivity is a central focus of this chapter. We consider the hypothesis that stress-induced premature senescence is triggered by signals arising directly from the photosynthetic apparatus, which can be regarded as a sensor of environmental change. Imbalances in electron flow between the photosystems and between electron transport and carbon assimilation can generate a range of oxidative and reductive signals. The activation of membrane-associated protein kinase pathways and singlet oxygen-associated signalling pathways has a profound effect on gene expression in the nucleus (Pfannschmidt 2010; Pogson et al. 2008).

7.2 Senescence-Associated Decline in Photosynthesis

Senescence is the final phase of leaf development, culminating in death of the organ. During senescence, photosynthesis declines due to the degradation of chlorophylls and proteins involved in photosynthetic reactions (Krupinska and Humbeck 2004). Senescence follows a sequential programme in cereals that is related to plant morphology. The primary foliage leaf is the first to undergo senescence, while the flag leaf is the last to show senescence symptoms. A main purpose of senescence is to remobilise valuable nutrients from older leaves for recycling and use in younger leaves or for grain filling. The period of leaf senescence represents something of a dilemma of the efficient operation of photosynthesis. It requires remarkable precision in targeted degradation of specific components while allowing others to continue to function without interruption. Photosynthetic proteins are degraded in order to remobilise nitrogen, but the overall integrity and functionality of the photosynthetic processes have to be maintained in order to provide ATP and reductant to drive senescence-associated degradation processes within the organelle (Krupinska and Humbeck 2004). A continuous reorganisation of the residual photosynthetic units is required in order to limit singlet oxygen formation in PSII and superoxide production at PSI, which might otherwise alter the pattern of nitrogen remobilisation. The reorganisation capacity has to remain high to ensure that the efficiency of photosynthesis remains largely unchanged until the final stages of senescence. The photosynthetic efficiency of wheat leaves declines during senescence when expressed on a leaf area basis, but it remains high throughout senescence when expressed relative to the abundance of chlorophyll (Camp et al. 1982). The kinetics of thylakoid membrane protein degradation are different from those of stromal proteins, such as the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Moreover, some thylakoid membrane proteins are much more stable during senescence than others (Krupinska and Humbeck 2004; Tang et al. 2005). Such findings provide evidence for the operation of different temporal and spatial regulatory mechanisms that control the degradation of the various components of the photosynthetic apparatus.

7.2.1 Rubisco Degradation

About 70 % of the nitrogen in chloroplasts is contained in the Rubisco proteins. Hence, Rubisco is an important nitrogen store. The regulated degradation of this enzyme protein is an essential step in nitrogen remobilisation to growing leaves, seeds and fruits. The degradation of Rubisco is a complex and poorly understood process. Rubisco can be degraded by different pathways under optimal and stress conditions. These can incorporate ROS-dependent steps, a range of proteolytic enzymes and subcellular compartments in addition to the chloroplasts (Feller et al. 2008). Perhaps, the most important pathway involves the cells' vesicular

trafficking system in which Rubisco-containing bodies (RCBs) transport stromal proteins including Rubisco to the vacuole for degradation (Ishida et al. 2008; Prins et al. 2008; Wada et al. 2009). The RCBs transport pathway resembles autophagy, which is a ubiquitous proteolytic protein degradation pathway (Izumi and Ishida 2011; Izumi et al. 2010). However, unlike autophagy, which is induced during senescence and in response to nitrogen deficiency, RCBs appear to be present at all stages of leaf development. RCBs are pinched off from chloroplasts or stromules (Prins et al. 2008). Hence, Rubisco mobilisation can occur without any prior degradation in the chloroplasts and stromal proteins can be degraded without a change in the number of chloroplasts, which remains constant in leaves until late in senescence (Feller et al. 2008). RCB production is regulated by environmental factors such as the nutrient status (Izumi and Ishida 2011; Izumi et al. 2010). For example, depletion of soluble sugars promotes RCB production while sugar accumulation tends to suppress it (Izumi et al. 2010; Izumi and Ishida 2011). The constitutive expression of the rice cystatin OC-1 in transgenic tobacco plants delays senescence and causes Rubisco accumulation particularly in older leaves (Prins et al. 2008). Such observations might suggest that a substantial proportion of the Rubisco protein is transported from the chloroplasts in RCBs that contain Rubisco degrading proteases, which will degrade the proteins (Feller et al. 2008; Prins et al. 2008).

7.2.2 Degradation of the Thylakoid Membrane System

A second major source of chloroplast nitrogen are the thylakoid membrane proteins, particularly the apoproteins of light-harvesting complex that are associated mainly with photosystem II (PSII) in the grana stacks. The thylakoid membrane of higher plant chloroplasts can be enriched in photosystem II (PSII) and light-harvesting complexes facilitating folding into grana stacks or in regions existing as unstacked stroma thylakoids (Anderson 1999). The unstacked stroma-facing thylakoids contain photosystem I (PSI), the cytochrome b6/f complex and the ATP synthase (Anderson and Andersson 1981). Little information is available on how the lateral heterogeneity in protein and lipid composition is changed during senescence (Anderson 1999; Anderson and Andersson 1982). Chlorophyll b is bound to the apoproteins of the light-harvesting complexes that are enriched in the regions of the grana stacks. The light-harvesting antenna complexes transfer light energy to the reaction centre complexes that contain only chlorophyll a molecules. Changes in the chlorophyll a/b ratio of leaves occurring during leaf senescence can give information on the mechanism and sequence of thylakoid membrane degradation. No changes in the chlorophyll a/b ratio will be observed if chloroplasts are ‘digested’ before conversion into gerontoplasts, but alternatively, if the composition of the thylakoid membrane proteins is changed during senescence, this should be reflected in changes in the chlorophyll a/b ratio. In studies

where the chlorophyll a/b ratio has been used as an indicator of senescence-associated chloroplast protein degradation, the leaf chlorophyll a/b ratio decreased because the stromal thylakoids are degraded before the grana thylakoids. For example, such changes have been reported in trees showing autumnal senescence (Wolf 1956) and also in crop plants such as rice (Kura-Hotta et al. 1987; Tang et al. 2005). Ultrastructural studies have also shown that grana thylakoids persist longer than stroma thylakoids during senescence, an observation that is consistent with the higher stability of the major light-harvesting complex (LHCII) and the measured decreases in chlorophyll a/b ratios. The early degradation of stroma thylakoids might imply that PSI is degraded before PSII. However, the resynthesis of PSII complexes in the repair cycle occurs in the stroma thylakoids and so the early degradation of stroma thylakoids will also limit the replacement of damaged PSII complexes within the grana thylakoids. This feature may explain some inconsistencies in studies concerning the sequence of degradation of the two photosystems during senescence (Krupinska and Humbeck 2004). Most studies report that an early loss of PSI was indeed observed (Miersch et al. 2000; Tang et al. 2005), but others suggest that PSI is more stable than PSII (Hilditch et al. 1986). Interestingly, the modern high yield barley variety cv Lomerit displays a rather different sequence of events in which the degradation of the grana precedes that of the stroma thylakoids (Krupinska et al. 2012). Moreover, the chlorophyll a/b ratio of the Lomerit flag leaves increased from 3.0 to 6.0 during senescence (Krupinska et al. 2012). In contrast to older barley varieties, the abundance of LHCII proteins declined in parallel with the abundance of the Rubisco large subunit in Lomerit leaves while gerontoplasts with long single or paired thylakoids were observed (Krupinska et al. 2012). The pathway of chloroplast degradation observed in the barley Lomerit cultivar might have at least two physiological consequences. Firstly, the parallel degradation of LHCII and PSII core proteins might prevent imbalances in the excitation of the photosystems and thereby might minimise the risk for enhanced singlet oxygen generation in PSII and photoinhibition. Secondly, the specific energy requirements for chloroplast breakdown might be met if loss of PSII activity is accompanied by enhanced cyclic electron flow around PSI. Enhanced rates of cyclic electron transport around PSI have been reported in senescence and in the leaves of plants exposed to stress (Rumeau et al. 2007). A higher ratio of production of ATP relative to reductant by the thylakoid electron transport system might be advantageous during senescence in order to support the ATP-dependent enzymes of chlorophyll degradation as well as lipid and protein remobilisation. Moreover, photosynthetic performance might be kept at a high level if the stroma thylakoids with the ATP synthesis and PSII repair machinery could be maintained for longer.

The degradation of LHCII could serve to decrease the excitation pressure on PSII and hence minimise the possibility of triplet chlorophyll and singlet oxygen formation. Changes in the balance of singlet oxygen to superoxide and hydrogen peroxide might have a profound effect on gene expression patterns as the different forms of reactive oxygen can act antagonistically in the regulation of different suites of defence genes. Photoinhibition can lead to the selective activation of suites

of singlet oxygen-responsive nuclear genes while at the same time repressing hydrogen peroxide-responsive genes. However, none of the forms of reactive oxygen produced in chloroplasts operate in isolated signalling pathways, but rather they act as part of a complex signalling network that integrates information from metabolism and the environment. Observations of singlet oxygen and hydrogen peroxide/superoxide production in flag barley leaves measured by electron spin resonance (ESR) spectroscopy suggest that these metabolites are generally much less abundant in the Lomerit cultivar introduced in 2001 than in the older variety (cv. Carina), which was introduced 30 years earlier (Krieger-Liszkay, Bilger and Krupinska, unpublished results). In the elder variety, hydrogen peroxide/superoxide levels were more than threefold higher in flag leaves during senescence, consistent with earlier observations in *Arabidopsis thaliana* that showed that senescing leaves have higher ROS levels (Zimmermann and Zentgraf 2005) and pea (Vanacker et al. 2006). A key question therefore concerns whether the ratio of singlet oxygen to hydrogen peroxide/superoxide signalling changes during senescence. The differences in hydrogen peroxide/superoxide levels in the senescing flag leaves of the different barley cultivars are certainly intriguing and show that plant breeding has resulted in the production of the new cultivars that are better able to maintain low ROS levels during senescence.

7.3 Reactive Oxygen Production and Antioxidative Systems

Leaf senescence occurs in an age-dependent manner during development, but it can also be triggered or accelerated by exposure to abiotic and biotic stresses such as extreme temperatures, mineral deficiency, drought or pathogen attack (Buchanan-Wollaston et al. 2003; Gepstein et al. 2003; Lim et al. 2007; Rosenwasser et al. 2011). Natural and stress-induced senescence involves decreased expression of photosynthesis-associated genes (PAGs) and other senescence downregulated genes (SDGs) and increased expression of senescence-activated genes (SAGs; Buchanan-Wollaston et al. 2003; Gepstein et al. 2003). The synergistic co-activation of nonspecific stress-responsive pathways that are induced under abiotic and biotic stress conditions involves enhanced ROS production and oxidative signalling (Bartoli et al. 2013). Redox signalling is coordinated with the hormone signalling network involving ethylene (ET), salicylic acid (SA), abscisic acid (ABA) and jasmonates (Bostock 2005; Foyer and Noctor 2009; Fujita et al. 2006). Many hormones promote ROS production as second messengers in signalling pathways often through the activation of NADPH oxidases, which are also involved in the hypersensitive response to pathogens (Bartoli et al. 2013; Grant and Loake 2000). It has long been accepted that oxidative signalling also plays a key role in natural and stress-induced senescence (Guo and Crawford 2005; Guo and Gan 2005; Miao et al. 2004; Navabpour et al. 2003). Moreover, the *oresara*

(long living) mutants of *A. thaliana*, which exhibit delayed natural senescence, have reduced stress sensitivity (Woo et al. 2001, 2004). While such studies link oxidative signalling to the control of leaf longevity, the analysis of *oresara* mutants has not shown that higher stress tolerance arises from enhanced antioxidant enzyme activities (Woo et al. 2002, 2004).

The synthesis and abundance of the major low molecular weight antioxidant, L-ascorbic acid (vitamin C), decreases during leaf development (Bartoli et al. 2000, 2005; Kingston-Smith et al. 1997; Pignocchi and Foyer 2003; Vanacker et al. 2006). Mutants that have defects in the ascorbate synthesis pathway of such as *vitamin c-1* (*vtc1*), which has a mutation in GDP-mannose pyrophosphorylase, exhibit higher expression of some *SAG* transcripts. SA accumulates to high levels in these mutants, and they show constitutive expression of pathogen-responsive genes (Barth et al. 2004; Kerchev et al. 2011; Pastori and del Rio 1994; Pavet et al. 2005).

While it is clear that ROS and antioxidants are key players in redox signalling pathways that are important in natural and stress-induced senescence programmes, much remains uncertain about the origins of the redox signals and their relationships to hormonal signalling both in salvage pathways and in the SA-induced cell death programme. The characterisation of these processes, the identification of components of the redox network and the elucidation of their roles will lead to new molecular tools that will enable better control of senescence processes and even extend leaf lifespan.

7.4 Chloroplasts as Environmental Sensors

In nature plants grow under fluctuating environmental conditions, requiring frequent acclimation of photosynthesis and other metabolic processes for optimal growth. If not regulated appropriately, the photosynthetic electron transport system uses redox signals as direct and dynamic means to regulate multiple chloroplast phenomena. Hence, chloroplasts are sensors of the environmental changes, fulfilling key roles in the regulation of plant growth and development in relation to environmental cues. Disruption of essential plastid functions impairs crucial stages in plant development, e.g. embryogenesis (Inaba and Ito-Inaba 2010). In addition to reactive oxygen species, chloroplasts produce various stress hormones such as ABA and strigolactones that elicit plant defence responses (Bouvier et al. 2009). The sensor function of chloroplasts is intrinsically linked to photosynthesis and its regulation. During senescence, like at all other stages of leaf development, the capacities for ATP and reductant production by the electron transport system are closely aligned to the energy needs of metabolism. Photosynthetic control of electron flow balances the provision of ATP and reductants (NADPH, reduced ferredoxin) with their utilisation, predominantly in carbon metabolism. This involves the regulated activation of Benson-Calvin cycle enzymes and precise regulation of the balance between noncyclic and cyclic electron flow pathways, as well as longer-term adjustments in chloroplast structure and composition (Foyer

et al. 2012). The photosynthetic electron transport chain contains an NADH dehydrogenase (NDH) complex that is homologous to the mitochondrial complex I. The NDH complex can associate with PSI in order to redirect electron flow and prevent over-reduction of the stroma by transporting electrons from PSI to the plastoquinone pool via NADPH. It is therefore considered to function in cyclic electron flow and in chlororespiration, in which the plastoquinone pool is reduced through the action of the NDH complex. It was originally suggested that the plastoquinone pool could also be oxidised by oxygen through the action of an oxidase, but no such oxidase protein has yet been identified. The majority of photosynthetic electron flow follows a noncyclic pathway from water to NADP, but cyclic electron flow around photosystem I (PSI) makes an important contribution to the overall regulation of electron transport by allowing ATP generation without the net production of reductant. The balance between noncyclic and cyclic electron flow pathways varies in response to changes in environmental conditions, particularly in stress situations, where CO_2 becomes limiting and metabolic needs require that the ratio of ATP to NAPDH production is continuously adjusted in order to minimise singlet oxygen production in PSII and superoxide production in PSI (Foyer et al. 2012). Studies involving chlorophyll fluorescence quenching analysis in senescent leaves suggest that the non-photochemical quenching (NPQ) component is increased (Dai et al. 2004). The requirement for greater xanthophyll cycle activity and thermal energy dissipation in PSII therefore increases during senescence of wheat leaves (Lu et al. 2003). This suggests that more excitation energy has to be dissipated as heat in order to decrease the probability of singlet oxygen production and maintain the photosynthetic function during senescence.

Singlet oxygen, superoxide and hydrogen peroxide produced in the chloroplast contribute to the repertoire of signals that regulate gene expression in relation to changes in the environmental and metabolic triggers. Reductive and oxidative signalling pathways are important in chloroplast to nucleus communication that regulates nuclear gene expression to ensure the stoichiometric assembly of plastid protein complexes and to initiate macromolecular reorganisation in response to environmental cues. The expression of nuclear-encoded photosynthetic genes can be either downregulated or upregulated in response to changes in the redox status of the chloroplast (Fey et al. 2005).

Little is known about how chloroplast to nucleus signalling (retrograde signalling) is achieved in senescing leaves, but it is possible that the repertoire of ‘retrograde signals’ includes metabolites that accumulate to a certain threshold that is recognised by the cell as a ‘metabolite signature’, which then triggers changes in gene expression (Pfannschmidt 2010). For example, chlorophylls are degraded during leaf senescence and the catabolites arising from this process could act as chloroplast to nucleus signals. However, many chlorophyll catabolites such as phaeophorbide also produce singlet oxygen that in excess can trigger genetically programmed cell suicide pathways (Pruzinska et al. 2007). While such metabolite signatures are by their nature highly complex, there is no doubt that they can convey information to the nucleus that complements information transmitted by the chloroplast kinase system, via protein phosphorylation and dephosphorylation. This

mainly concerns imbalances in energy supply and energy consumption within the photosynthetic electron transport chain and plays a key role in the adaptation of plants to environmental changes such as in temperature (Huner et al. 1996) or light intensity (Soitamo et al. 2008) as well as to changes in chloroplast composition occurring during senescence. Thylakoid membrane composition may be influenced by differences in the stabilities of the light-harvesting complexes such as CP43, CP47, LHCI and LHCII (Tang et al. 2005). However, a much greater understanding of the chloroplast to nucleus retrograde signalling pathways and how they function during leaf senescence is required in order to improve photosynthesis and radiation use efficiency. It is worth noting that although the phenomenon of chloroplast to nucleus retrograde signalling has been known for over 30 years, no pathways of retrograde signalling that alter the action of known transcription factors have as yet been completely identified. To date, only four transcription factors (WHIRLY1, PTM, a chloroplast envelope-bound plant homeodomain transcription factor, abscisic acid-insensitive ABI4 and bZIP16) have been shown to function in retrograde signalling, but their target genes and mechanisms of action are still largely unknown.

Any imbalance in excitation of PSI and PSII caused, for example, by changes in light intensity or quality will lead to alterations in the redox state of electron transport carriers, particularly the plastoquinone pool, and also influence the production of reactive oxygen species (Mühlenbock et al. 2008; Pfannschmidt et al. 2009). In addition, chloroplast signals do not operate in isolation, but rather they are integrated with the cellular signalling network. Hence, exposure to high light can trigger gene expression patterns leading to enhanced pathogen responses (Karpinski et al. 2003). Such chloroplast signalling pathways can involve induction of the synthesis of signalling compounds such as ABA that enhance defences towards specific pathogens (Bouvier et al. 2009). An increasing body of literature evidence supports a role for chloroplasts in plant stress responses and the induction of pathogen-associated molecular patterns (PAMPs) that are recognised by cells of the plant innate immune system. Innate immunity is an antigen-nonspecific defence mechanism that links photosynthesis and pathogen defence reactions (Mateo et al. 2006; Mühlenbock et al. 2008). Silencing of a component of PSII in plants infected with tobacco mosaic virus (TMV) led to a 10-fold higher virus accumulation (Abbink et al. 2002). Conversely, TMV infection had a negative effect on PSII function and caused chlorosis (Lehto et al. 2003).

7.5 Plastidial Signalling During Senescence

Studies on senescence in the model plant *A. thaliana* (Hensel et al. 1993) and in barley flag leaves collected from field-grown plants (Humbeck et al. 1996) have clearly shown that photosynthetic activity declines prior to the upregulation of major SAG expression. Such observations suggest that nuclear gene expression

during senescence is controlled by signals originating from chloroplasts, as proposed originally by Hensel and co-workers (Hensel et al. 1993).

As discussed above, the chloroplast signalling pathways (Fig. 7.1) involve metabolites such as sugars (Leister 2005), intermediates of the tetrapyrrole biosynthesis, reactive oxygen species (Apel and Hirt 2004) as well as protein kinase pathways that are subject to regulation from redox signals associated with the redox state of the plastoquinone pool (Pfannschmidt et al. 2009; Pogson et al. 2008). Plastoquinone oxidation controls the rate of transcription of genes encoding reaction centre apoproteins as well as the post-translational regulation of photosynthetic proteins by phosphorylation. Together with singlet oxygen and hydrogen peroxide, plastoquinone oxidation participates in chloroplast to nucleus retrograde signalling pathways that involve protein kinase signalling cascades (Foyer et al. 2012). In addition, a range of other regulatory proteins that participate in chloroplast to nucleus signalling are found inside (Inze et al. 2012; Isemer et al. 2012) and on the surface (Shang et al. 2010; Sun et al. 2011) of chloroplasts. Other plastidial factors that are intimately involved in chloroplast communication are the senescence-promoting hormones such as jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA), whose production is probably coordinated with changes in the efficiency of the photosynthetic apparatus.

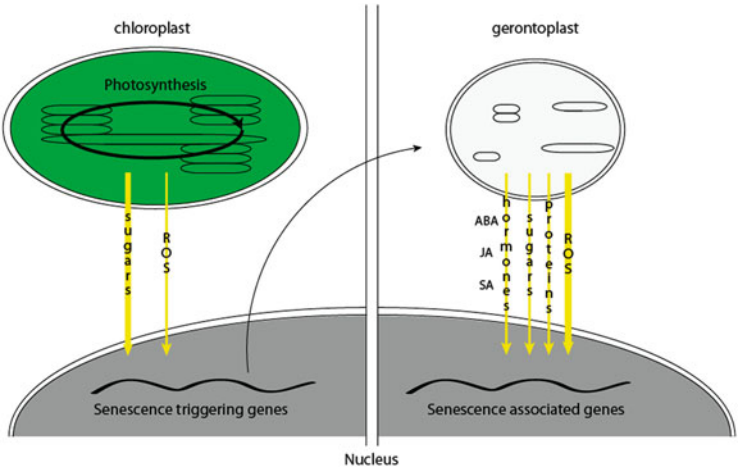

Fig. 7.1 Control of senescence-related gene expression by factors originating from chloroplasts. Gene expression in the nucleus is controlled by chloroplast signals such as reactive oxygen species and metabolites such as sugars and redox changes. During senescence the level of reactive oxygen species increases, whereas the level of sugars exported from plastids declines. Moreover, during senescence plastids produce the hormones ABA, JA and SA known to induce expression of senescence-associated genes. Regulatory proteins originating from chloroplasts are proposed to be involved in communication of the functional changes in the plastid to the nucleus

7.5.1 Reactive Oxygen Species and Redox Signals

In photosynthetic electron transport reducing equivalents are passed from PS I to NADP via ferredoxin to ferredoxin-dependent NADP+ reductases to produce NADPH, providing the reducing power to drive carbon fixation and other assimilatory processes. Photosynthetic electron transport also produces reductive signals (reducing equivalents) that are also transferred for regulatory purposes from ferredoxin via ferredoxin-dependent thioredoxin reductase to several chloroplast thioredoxins (Trxs). The reductive signal is then used by the Trxs to reduce disulphide bonds in regulatory sites of target chloroplast proteins, thereby modulating their activities. Trx-regulated proteins in the chloroplasts are generally oxidised in the dark and are reduced upon illumination (Schurmann and Buchanan 2008). Trxs not only regulate the activation states of enzymes in the chloroplasts, but they also interact with potential signalling proteins such as 2-Cys peroxiredoxins and glutaredoxins (Foyer et al. 2012).

Changes in photosynthetic electron transport and energy metabolism are also transmitted by oxidative signalling pathways that regulate gene transcription and post-transcriptional processing (Foyer et al. 2012). Of these, singlet oxygen is a major oxidative signal that leads to substantial reprogramming of gene expression and triggers programmed cell death (Gadjev et al. 2006). The two plastid proteins EXECUTER1 and EXECUTER2 are required for the translocation of singlet oxygen-derived signals arising from the plastid to the nucleus (Lee et al. 2007; Wagner et al. 2004). Singlet oxygen contributes to the complex cellular signalling network that integrates various extra- and intracellular cues (Baruah et al. 2009), but its role in senescence remains to be characterised. The expression of several sets of genes is differentially regulated by singlet oxygen and other ROS such as superoxide and hydrogen peroxide (Op den Camp et al. 2003). Singlet oxygen can also act antagonistically in the regulation of gene expression to hydrogen peroxide, which has long been considered to be an important signalling molecule triggering leaf senescence (Zimmermann and Zentgraf 2005). Hydrogen peroxide induces JUNGBRUNNEN1 (JUB1), a NAC transcription factor that negatively controls senescence (Wu et al. 2012). Overexpression of *JUB1* decreases hydrogen peroxide levels and markedly extends leaf longevity, as well as promotes tolerance to various abiotic stresses. The JUB1 transcription factor is therefore an important regulator of cellular redox homeostasis linking oxidative signalling to the control of senescence and the downstream activation of genes such as DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A (*DREB2A*) that regulate tolerance to abiotic stress. *DREB2A* expression increases during senescence and regulates the water deficit-inducible expression of target genes. Hydrogen peroxide can also induce other NAC factors that show senescence-dependent expression of genes such as *ORE1*, *NAP* and *ORS1* (Balazadeh et al. 2008, 2010).

Mutations in the *Arabidopsis CPR5/OLD1* gene result in defective responses to multiple hormonal and other signalling molecules leading to spontaneous lesion formation and premature senescence linked to enhanced oxidation (Jing

et al. 2008). However, only a subset of ROS-induced transcription factors were increased in *cpr5* mutants, indicating that very specific ROS pathways were activated (Jing et al. 2008). Analysis of changes in the redox state of different subcellular compartments during dark-induced senescence showed that oxidation occurred first in mitochondria followed by an oxidation of peroxisomes and that these changes occurred before any senescence symptoms were apparent. In contrast, the chloroplasts became more reduced early in senescence followed by a subsequent increase in oxidation, while the redox state of the cytoplasm was largely unchanged during dark-induced senescence (Rosenwasser et al. 2011). These changes were accompanied by increases in the expression of ROS-related genes with patterns that implicate mitochondrial processes as important activators of the senescence programme (Rosenwasser et al. 2011). Transgenic tobacco plants with impaired chloroplast NDH complex function show delayed leaf senescence (Zapata et al. 2005). An increase in NDH activity during senescence has been suggested to coincide with a reduced electron transporter availability in the thylakoid membrane (Zapata et al. 2005). The expression of genes encoding subunits of the NDH complex increases with leaf age (Casano et al. 1999), whereas expression of genes for chloroplast SOD (Casano et al. 1994, 1999) and other antioxidants tends to decrease during senescence (Kingston-Smith et al. 1997).

7.5.2 Sugar Signalling

Plant sugar signalling operates within the complex network of plant hormone signalling pathways that operate during senescence (Balazadeh et al. 2010; Rolland and Sheen 2005). Sucrose and hexoses are strong repressors of photosynthetic gene expression and their accumulation can lead to premature senescence. Carbohydrate-mediated repression of the expression of photosynthesis genes serves to resolve imbalances in carbon and nitrogen metabolism during senescence (Price et al. 2004). Carbohydrate accumulation also induces genes encoding proteins involved in nitrogen assimilation (Price et al. 2004). Of the genes whose transcription is specifically regulated by sucrose (Wind et al. 2010), the bZIP11 transcription factors are important in regulation of growth (Delatte et al. 2011; Hanson and Smeekens 2009; Wingler et al. 2012). The nuclear hexokinase1 (HXK1) complex controls glucose-mediated regulated expression of photosynthesis genes through binding to target gene promoters in cooperation with other proteins (Baena-Gonzalez 2010). This hexokinase-mediated sugar-sensing pathway is linked to protein serine/threonine kinases including the sucrose nonfermenting-1-related protein (SNF1) kinase cascades that are activated by starvation conditions and inhibited by high energy and carbon supply. SnRK1 is a central stress-integrating kinase that regulates genes that have diverse functions in metabolism, growth and development. Trehalose metabolism has a significant impact on SnRK1 activity (Zhang et al. 2009). The regulation of the SnRK1 pathway during leaf senescence is largely unexplored, but inhibition of SnRK1 activity by trehalose-6-phosphate may

be an important factor in the control of growth by bZIP transcription factors that regulate a range of basic developmental processes (Delatte et al. 2011).

7.5.3 Hormones

Senescence is controlled by the balance between the senescence-promoting hormones such as ET and ABA and senescence-retarding hormones such as cytokinins (Schippers et al. 2007). Three major senescence-promoting hormones—ABA, JA and SA—can be regarded as plastid signalling compounds (Fig. 7.1), because they are partially or completely synthesised in chloroplasts: light regulates the expression of genes involved in the synthesis of hormones such as ABA (Soitamo et al. 2008), indicating a link between photosynthesis and chloroplast localised steps of hormone biosynthesis.

Abscisic Acid (ABA) The initial first steps of ABA biosynthesis take place in the plastids with violaxanthin and/or neoxanthin as substrates (Cutler and Krochko 1999). During the senescence of crop plants such as oat (Gepstein and Thimann 1980), rice (Philosoph-Hadas et al. 1993) and maize (He et al. 2005), the water potential of the mesophyll cells declines in parallel with increases in the ABA level. Genes encoding key enzymes in ABA biosynthesis such as NCED are upregulated by exposure to drought (Seiler et al. 2011) and during senescence (Buchanan-Wollaston et al. 2005). Exogenously applied ABA promotes senescence and hence ABA is regarded as a senescence trigger (Gepstein and Thimann 1980; Quiles et al. 1995). However, in a recent study, the senescence-promoting effect of ABA was only observed in old *A. thaliana* leaves and not in the younger leaves (Lee et al. 2007). ABA, which produces hydrogen peroxide as a second messenger by activation of NADPH oxidases to induce stomatal closure in stress situations, may also contribute to hydrogen peroxide production during senescence (Hung and Kao 2004). The ABA content of leaves is enhanced in response to a range of environmental stress conditions such as high salinity, low temperatures and drought, all of which trigger premature senescence. ABA is therefore considered to be important in the regulation of stress-induced senescence (Yang et al. 2003).

Jasmonic Acid (JA) JA is synthesised in chloroplasts from linolenic acid. Lipoxygenases convert linolenic acid into hydroperoxylinolenic acid, from which a range of different metabolites is produced including cis(+)-12-oxophytodienoic acid (OPDA), which is the last component of the pathway in the chloroplasts. OPDA can be retained in the chloroplasts where it is used as a precursor of the signalling oxylipins in the chloroplasts. Alternatively, OPDA can be transported from the chloroplast to be further oxidised to JA in the peroxisomes. JA and related compounds regulate plant responses to wounding and necrotrophic pathogens (Devoto and Turner 2005). Moreover, JA can induce premature senescence leading to loss of chlorophyll and a reduction in Rubisco (Parthier 1990). While the *Coi1* Arabidopsis mutants that are defective in JA signalling do not show symptoms of

premature senescence, the expression of various senescence-associated genes is enhanced by JA (He et al. 2002).

Salicylic Acid (SA) SA is the 2-hydroxyl derivate of benzoic acid. Initially thought to be synthesised in the cytoplasm by the phenylpropanoid pathway, SA biosynthesis is now considered to take place predominantly in plastids (Strawn et al. 2007; Wildermuth et al. 2001). Increasing evidence suggests that SA has many functions in plants in addition to the well-known roles in pathogen defence signalling. For example, SA has been implicated in acclimation to high light and in regulation of the redox homeostasis (Mateo et al. 2006; Mühlenbock et al. 2008). SA levels increase during senescence (Morris et al. 2000). Transgenic Arabidopsis plants overexpressing the bacterial *NahG* gene encoding an SA hydrolase are compromised in SA signalling and also show delayed senescence, as determined by leaf chlorophyll and photosynthetic capacity (F_v/F_m), but they showed patterns of dark-induced senescence similar to wild-type plants (Buchanan-Wollaston et al. 2005). Many SA-responsive genes are upregulated during natural senescence, but they are not induced by dark-induced senescence (Buchanan-Wollaston et al. 2005; Graaff et al. 2006; Morris et al. 2000). It has therefore been proposed that SA is primarily involved in control of programmed cell death at the later stages of the senescence process (Buchanan-Wollaston et al. 2003).

7.5.4 Transcription Factors Sequestered by Chloroplasts

A number of DNA-binding proteins are predicted to be targeted to chloroplasts and also to the nucleus, thereby offering new possibilities for the coordination of DNA-associated activities in both compartments (Schwacke et al. 2007; Wagner and Pfannschmidt 2006). WHIRLY1 was the first transcription factor that was shown to be dual-targeted to plastids and the nucleus of the same cell within a barley leaf (Grabowski et al. 2008). Moreover, the plastidial form of WHIRLY1 can be translocated from plastids to the nucleus (Isemer et al. 2012). Transgenic barley plants with a knockdown of the *WHIRLY1* gene expression display delayed senescence (Krupinska, unpublished results). Other transcription factors with predicted plastid-targeting include several NAC domain and WRKY transcription factors (Schwacke et al. 2007). Several members of these groups of transcription factors either affect or are associated with leaf senescence (Balzadeh et al. 2008; Christiansen et al. 2011; Wu et al. 2012). In *A. thaliana*, the NAC factor ANAC102 is targeted to chloroplasts, but it is translocated to the nucleus when the redox balance of chloroplasts is perturbed, where it regulates stress-related gene expression (Inze et al. 2012).

Other transcription factors such as WRKY40 that are involved in the regulation of senescence can also bind to the surface of the chloroplast envelope. After treatment with ABA, the *A. thaliana* WRKY40 is no longer found in nucleus, but it is bound to subunit H of magnesium chelatase, which is located on the outer

surface of the chloroplasts (Shang et al. 2010). Another potential senescence regulator found on the outer chloroplast membrane, Tsip1, dissociates from the surface after SA treatment and is then found in the nucleus, where it activates stress-responsive genes (Ham et al. 2006). These findings suggest that some functions of hormones such as ABA and JA that originate in chloroplasts might involve a redistribution of regulatory proteins within the cell. Such a scenario may occur during senescence when intracellular compartmentation is changed.

7.6 Photosynthesis-Based Approaches to Increase Crop Productivity

7.6.1 Relationship Between Photosynthetic Duration and Biomass Yield

It has long been postulated (Thomas and Stoddart 1980) that crop yields might be improved by delaying or modulating leaf senescence. Such hypotheses assume that the period of maximal photosynthetic activity can be extended by delaying senescence and that this in turn should lead to higher biomass production. However, this assumption has been questioned with regard to cereal grain yields, where yield is primarily determined by the number of grains per area and yield is only affected to a limited extent by grain size (Fischer 2007) (Fig. 7.2). Thus, sink limitations on yield mean that this parameter is determined by physiological processes that occur in the middle of the growing season, i.e. up to and around anthesis. At this stage, the number of fertile florets is already established. Hence, the number of grains will not

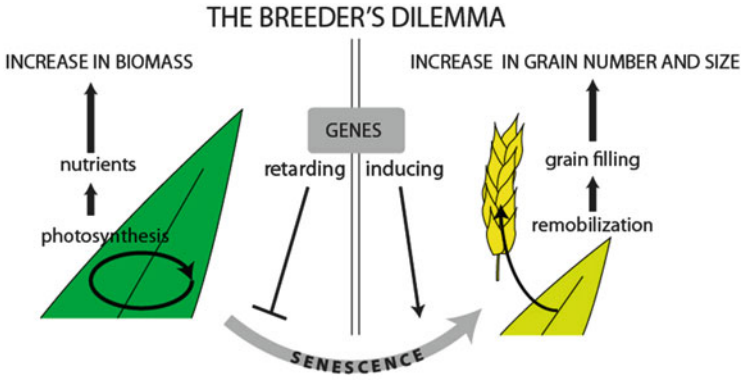

Fig. 7.2 The breeder's dilemma. On one hand retardation of senescence is required for breeding crop plants with more biomass. On the other hand senescence is required for efficient nutrient remobilisation. An increase in grain number and size hence might be only achieved at the expense of biomass production

be changed by an extended period of photosynthesis, especially under optimal growing conditions (Reynolds et al. 2005). However, increases in radiation use efficiency obtained by genetic manipulation of the senescence programme should be accompanied by other adaptations in the whole-plant physiology. Stay-green phenotypes that have a sustained long period of high photosynthetic capacity could increase grain numbers per area as well as result in a greater number of stronger sinks that would again enhance photosynthetic rates (Luo et al. 2009). Some stay-green wheat cultivars have larger grains (Chen et al. 2010). Moreover, in durum wheat cultivars with stay-green characteristics, the grain filling period is extended (Gebeyehou et al. 1982).

The extended grain filling period in stay-green cereal cultivars can however lead to other constraints, for example, in relation to optimal regional maturation and harvest time. Hence, with the exception of maize, there are as yet no striking examples of how delayed senescence can be used as a driver for yield increase in small grain cereals. The improved productivity of modern maize cultivars is associated to a large degree with delayed senescence characteristics compared to older cultivars (Ding et al. 2005; Tollenaar and Lee 2006). Since maize is a relatively new crop species in terms of growth at northern latitudes, the delayed senescence characteristics might reflect a regional adaptation in life span regulation, brought about by classic plant breeding approaches. It is possible that optimal life span control may have already been attained in other temperate cereals, such as barley and wheat, during their long history of domestication.

7.6.2 Functional Stay-Green Plants

During leaf senescence photosynthetic function can either decline in parallel with chlorophyll or can be more stable than chlorophyll content of the leaves (Fig. 7.3). On the other hand, in 'cosmetic' stay-green lines, chlorophyll content stays high, while photosynthesis declines (Hörtensteiner 2009). There is however an increasing number of reports demonstrating that cultivars with delayed senescence ('stay-green' phenotypes) characteristics, particularly in wheat, are functional stay-green plants having increased yields (e.g. Chen et al. 2011; Chen et al. 2010; Spano et al. 2003; and review by Gregersen et al. 2013). In some cases, this is accompanied by reductions in nitrogen remobilisation efficiency (Chen et al. 2010, 2011; Spano et al. 2003) and low harvest index (Gong et al. 2005). These findings illustrate some of the constraints imposed by the growth environment that can limit the productivity of a crop, making it difficult in practice to exploit any potential benefits offered by delayed senescence. A relationship between an extended green leaf area duration and yield has also been demonstrated in maize and sorghum cultivars (Ding et al. 2005; Vadez et al. 2011a). Recent publications have also reported that an enhanced performance of different barley cultivars under drought is associated with a stay-green phenotype, scored either visually or from chlorophyll content (Gonzalez et al. 2010; Vaezi et al. 2010). Enhanced

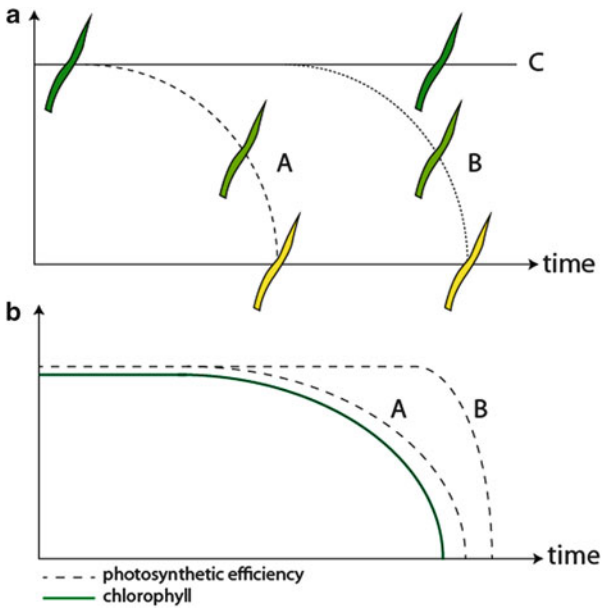

Fig. 7.3 Different patterns of senescence. (**a**) Senescence in leaves can proceed fast (A) or can be retarded (B). In some mutants chlorophyll content stays high until the final end of the leaf's life. (**b**) Chlorophyll content of leaves and photosynthetic efficiency may decline in parallel as shown for flag leaves of the barley variety Carina (A) (Humbeck et al. 1996). Photosynthetic efficiency of the remaining photosynthetic units could stay high during senescence when chlorophyll declines as shown for flag leaves of the barley variety Lomerit (B) (Krupinska et al. 2012)

performance under environmental stress conditions is often found to be a characteristic of stay-green cultivars in different species (Naruoka et al. 2012; Rivero et al. 2007). Such observations point to improvements in stress adaptation and physiology in at least some stay-green phenotypes.

Functional stay-green phenotypes observed in different species and cultivars are multigenic traits. Much remains uncertain about how chloroplast degradation is altered to produce stay-green phenotypes (Krupinska et al. 2012). Major breakthroughs are required in our current understanding of the regulation of senescence in order to identify crucial factors that can be incorporated into marker-assisted selection. Given the lack of current knowledge on this topic, it may be possible to increase productivity further through enhanced photosynthetic capacity and efficiency (Parry et al. 2011). Molecular approaches have already provided some candidate genes that allow dissection of QTLs or enable transgenic approaches to test genes with regard to delayed senescence and enhanced radiation use efficiency.

Hormone-regulated changes in gene transcriptional networks are crucial to the execution of the senescence programme. Hence, chloroplast degradation is influenced by a wide range of different factors that alter hormone signalling. For example, variations in transpiration efficiency related to root properties are the basis for stay-green in certain sorghum genotypes (Vadez et al. 2011b) and also in wheat lines that differ in the duration of green leaf area (Naruoka et al. 2012). In such cases, the stay-green phenotype is related to improved drought resistance. However, the link between transpiration and senescence regulation is far from trivial, because plant hormones such as ABA and cytokinins, which for a long time have been known to control transpiration processes (Mizrahi et al. 1970), also

regulate senescence (Schippers et al. 2007). A wide range of abiotic stress- and senescence-associated genes such as NAC transcription factors are regulated by ABA and other hormones (Christiansen et al. 2011; Cutler et al. 2010). Although the regulation of senescence shows a strong overlap with drought resistance, experiments in *A. thaliana*, in which the onset of senescence is delayed as a result of modulation of NAC transcription factors, show that there is a strong genetic regulation of the senescence process per se (Balazadeh et al. 2011; Guo and Gan 2006; Kim et al. 2009; Wu et al. 2012). This regulation is interconnected with the networks of hormonal and redox regulation, as illustrated by the hydrogen peroxide-dependent regulation of the expression of *JUB1*, which regulates longevity in Arabidopsis (Wu et al. 2012). However, in many cases it is not clear whether the regulation of senescence can be separated easily from earliness/lateness in the general plant development, e.g. in relation to flowering time. A delay in leaf senescence may also engender an extension of the whole life span of the plant from sowing to maturity.

7.6.3 Accelerating the Senescence Process

The suitability for agriculture of stay-green plants with an extended grain filling period is highly dependent on the growing regions. A crucial question therefore concerns the value of stay-green approaches that lead to an extension of the grain filling period for agriculture, particularly with respect to the constraints set by the length of the growth season in a given environment. Moreover, it may be beneficial to speed up, rather than delay, the senescence process when short growing seasons prevail (Brevis and Dubcovsky 2010; Iqbal et al. 2007; Uauy et al. 2006). For example, Iqbal et al. (2007) concluded that an ideal phenotype of spring wheat grown under high latitude conditions would involve a more rapid senescence process coupled to delayed anthesis and a higher grain fill rate resulting in higher grain protein contents. In the natural and landrace adaptation of cereal plants to Mediterranean conditions, where a hot and dry summer sets the upper limit for the growing season under nonirrigated conditions, an acceleration of the senescence process is necessary to promote remobilisation of nutrients (Hafsi et al. 2000). The early and fast senescence occurring under Mediterranean conditions has been exploited in modern breeding of durum wheat cultivars (Isidro et al. 2011). A rapid and intensive senescence process is required in order to achieve high grain protein contents in stressful environmental conditions. The importance of this trait is demonstrated by the association of accelerated senescence with high protein contents in *Gpc-B1* wheat lines (Uauy et al. 2006; Brevis and Dubcovsky 2010). Hence, the timing of senescence appears to have a stronger influence on crop performance with respect to the nitrogen/protein content of the maturing cereal seed than to the total biomass yield level.

Several examples of stay-green cereal cultivars further illustrate the ‘dilemma’ of the plant (Gregersen et al. 2008) (Fig. 7.2). As discussed above, it is possible to

delay senescence in stay-green phenotypes and to increase yield accordingly. However, delaying senescence is often achieved at the expense of nitrogen remobilisation and hence stay-green cultivars can exhibit relatively low grain protein contents (Chen et al. 2011). The functionality of the photosynthetic apparatus must stay high even during the phase of dismantling and degradation of the different chloroplast components as illustrated in Fig. 7.3b. To achieve this goal degradation processes need to be tightly coordinated even under stressful environmental conditions. A much more in-depth knowledge of the senescence-associated degradation of the photosynthetic apparatus and how chloroplast signals control overall plant performance during senescence is essential for the breeding of new varieties with improved senescence characteristics.

Conclusions

The senescence process is central to the remobilisation of nitrogen resources during the maturation of cereal crop plants such as barley. The analysis of stay-green lines and mutants has demonstrated that productivity of crop plants can be enhanced by prolonging the lifespan of the photosynthetic tissues. Future agriculture requires crop plants with highly stable productivity even under adverse environmental conditions. Selection programmes for improved barley varieties must couple enhanced productivity with high stress tolerance. The challenge for the future therefore is to tailor the timing and intensity of the senescence process according to the needs of the agricultural environment, such that not only the onset of senescence can be controlled but also the intensity and speed of the senescence process. In this way, yield increases can be achieved by the efficient remobilisation of nutrients under optimal, temperate climate conditions. In essence, the trait requirement is to combine the mid-late flowering time of temperate climates with the high-rate senescence programme typical of cereal plants from Mediterranean dry regions. Key to achieving this goal is the application of current and emerging knowledge on the regulatory genetic, biochemical and redox networks that control the stability, and subsequently dismantling, of the photosynthetic apparatus during senescence.

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References

Abbink TEM, Peart JR, Mos TNM, Baulcombe DC, Bol JF, Linthorst HJM (2002) Silencing of a gene encoding a protein component of the oxygen-evolving complex of photosystem II enhances virus replication in plants. Virology 295:307–319

Anderson JM (1999) Insights into the consequence of grana stacking of thylakoid membranes in vascular plants: a personal perspective. Aust J Plant Physiol 26:625–639

Anderson JM, Andersson B (1981) Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. Proc Aust Biochem Soc 14:124

Anderson JM, Andersson B (1982) The architecture of photosynthetic membranes – lateral and transverse organization. Trends Biochem Sci 7:288–292

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Physiol Plant Mol Biol 55:373–399

Austin RB, Bingham J, Blackwell RD, Evans LT, Ford MA, Morgan CL, Taylor M (1980) Genetic improvements in winter wheat yields since 1900 and associated physiological changes. J Agric Sci 94:675–689

Baena-Gonzalez E (2010) Energy signaling in the regulation of gene expression during stress. Mol Plant 3:300–313

Balazadeh S, Riano-Pachón D, Mueller-Roeber B (2008) Transcription factors regulating leaf senescence in *Arabidopsis thaliana*. Plant Biol 10:63–75

Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, Mehrnia M, Zanor MI, Kohler B, Mueller-Roeber B (2010) A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. Plant J 62:250–264

Balazadeh S, Kwasniewski M, Caldana C, Mehrnia M, Zanor MI, Xue GP, Mueller-Roeber B (2011) ORS1, an H2O2-responsive NAC transcription factor, controls senescence in Arabidopsis thaliana. Mol Plant 4:346–360

Balzadeh S, Parlitz S, Mueller-Roeber B, Meyer RC (2008) Natural developmental variations in leaf and plant senescence in Arabidopsis thaliana. Plant Biol 10:136–147

Barth C, Moeder W, Klessig DF, Conklin PL (2004) The timing of senescence and response to pathogens is altered in the ascorbate-deficient Arabidopsis mutant vitamin c-1. Plant Physiol 134:1784–1792

Bartoli CG, Pastori GM, Foyer CH (2000) Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. Plant Physiol 123:335–343

Bartoli CG, Guiamet JJ, Kiddle G, Pastori GM, Di Cagno R, Theodoulou FL, Foyer CH (2005) Ascorbate content of wheat leaves is not determined by maximal L-galactono-1,4-lactone dehydrogenase (GalLDH) activity under drought stress. Plant Cell Environ 28:1073–1081

Bartoli CG, Casalongue C, Simontacchi M, Marquez-Garcia B, Foyer CH (2013) Interactions between hormone and redox signalling pathways. Environ Exp Bot 94:73–88

Baruah A, Simkova K, Hincha DK, Apel K, Laloi C (2009) Modulation of (1)O(2)-mediated retrograde signaling by the PLEIOTROPIC RESPONSE LOCUS 1 (PRL1) protein, a central integrator of stress and energy signaling. Plant J 60:22–32

Bingham IJ, Blake J, Foulkes MJ, Spink J (2007) Is barley in the UK sink limited? Post-anthesis radiation interception, radiation-use efficiency and source-sink balance. Field Crops Res 101:198–211

Bostock RM (2005) Signal crosstalk and induced resistance: straddling the line between cost and benefit. Annu Rev Phytopathol 43:545–580

Bouvier F, Mialoundama AS, Camara B (2009) A sentinel role for plastids. In: Sandelius AS, Aronsson H (eds) The chloroplast. Springer, Berlin, pp 267–292

Brevis JC, Dubcovsky J (2010) Effects of the chromosome region including the Gpc-B1 locus on wheat grain and protein. Crop Sci 50:93–104

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D (2003) The molecular analysis of leaf senescence – a genomics approach. Plant Biotechnol 1:3–22

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J 42:567–585

Camp PJ, Huber SC, Burke JJ, Moreland DE (1982) Biochemical changes that occur during senescence of wheat leaves. Plant Physiol 70:1641–1646

Casano LM, Martin M, Sabater B (1994) Sensitivity of superoxide dismutase transcript levels and activities to oxidative stress is lower in mature-senescent than in young barley leaves. Plant Physiol 106:1033–1039

Casano LM, Martín M, Zapata JM, Sabater B (1999) Leaf-age and paraquat concentration-dependent effects on the levels of enzymes protecting against photooxidative stress. Plant Sci 149:13–22

Chen JB, Liang Y, Hu XY, Wang XX, Tan FQ, Zhang HQ, Ren ZL, Luo PG (2010) Physiological characterization of 'stay green' wheat cultivars during the grain filling stage under field growing conditions. Acta Physiol Plant 32:875–882

Chen CC, Han GQ, He HQ, Westcott M (2011) Yield, protein, and remobilization of water soluble carbohydrate and nitrogen of three spring wheat cultivars as influenced by nitrogen input. Agron J 103:786–795

Christiansen MW, Holm PB, Gregersen PL (2011) Characterization of barley (Hordeum vulgare L.) NAC transcription factors suggests conserved functions compared to both monocots and discots. BMC Res Notes 4:302

Cutler AJ, Krochko JE (1999) Formation and breakdown of ABA. Trends Plant Sci 4:472–478

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. In: Merchant S, Briggs WR, Ort D (eds) Annual review of plant biology, vol 61., pp 651–679

Dai J, Gao Y, Zou Q (2004) Changes in activity of energy dissipating mechanisms in wheat flag leaves during senescence. Plant Biol 6:171–177

Delatte TL, Sedijani P, Kondou Y, Matsui M, de Jong GJ, Somsen GW, Wiese-Klinkenberg A, Primavesi LF, Paul MJ, Schluepmann H (2011) Growth arrest by trehalose-6-phosphate: an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway. Plant Physiol 157:160–174

Devoto A, Turner JG (2005) Jasmonate-regulated Arabidopsis stress signalling network. Physiol Plant 123:161–172

Ding L, Wang KJ, Jiang GM, Liu MZ, Niu SL, Gao LM (2005) Post-anthesis changes in photosynthetic traits of maize hybrids released in different years. Field Crops Res 93:108–115

Feller U, Anders I, Mae T (2008) Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. J Exp Bot 59:1615–1624

Fey V, Wagner R, Brautigam K, Pfannschmidt T (2005) Photosynthetic redox control of nuclear gene expression. J Exp Bot 56:1491–1498

Fischer AM (2007) Nutrient remobilization during leaf senescence. In: Gan S (ed) Senescence processes in plants. Blackwell, Oxford, pp 87–107

Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid Redox Signal 11:861–905

Foyer CH, Neukermans J, Queval G, Noctor G, Harbinson J (2012) Photosynthetic control of electron transport and the regulation of gene expression. J Exp Bot 63:1637–1661

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol 9:436–442

Gadjev I, Vanderauwera S, Gechev TS, Laloi C, Minkov IN, Shulaev V, Apel K, Inze D, Mittler R, Van Breusegem F (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. Plant Physiol 141:436–445

Gebeyehou G, Knott DR, Baker RJ (1982) Rate and duration of grain filling in durum wheat cultivars. Crop Sci 22:337–34

Gepstein S, Thimann KV (1980) Changes in the abscisic acid content of oat leaves during senescence. Proc Natl Acad Sci U S A 77:2050–2053

Gepstein S, Sabehi G, Carp M-J, Hajouj T, Nesher MF, Yariv I, Dor C, Bassani M (2003) Large-scale identification of leaf senescence-associated genes. Plant J 36:629–642

Gong Y, Zhang J, Gao J, Lu L, Wang J (2005) Slow export of photoassimilate from stay-green leaves during late grain-filling stage in hybrid winter wheat (*Triticum aestivum* L.). J Agron Crop Sci 191:292–299

Gonzalez A, Bermejo V, Gimeno BS (2010) Effect of different physiological traits on grain yield in barley grown under irrigated and terminal water deficit conditions. J Agric Sci 148:319–328

Graaff E, Schwacke R, Schneider A, Desimone M, Flügge U-I, Kunze R (2006) Transcription analysis of Arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol 141:776–792

Grabowski E, Miao Y, Mulisch M, Krupinska K (2008) Single-stranded DNA binding protein Whirly1 in barley leaves is located in chloroplasts and nuclei of the same cell. Plant Physiol 147:1800–1804

Grant JJ, Loake GJ (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol 124:21–29

Gregersen PL, Holm PB, Krupinska K (2008) Leaf senescence and nutrient remobilisation in barley and wheat. Plant Biol 10:37–49

Gregersen PL, Culetic A, Boschian L, Krupinska K (2013) Plant senescence and crop productivity. Plant Mol Biol 82:603–622

Guo FQ, Crawford NM (2005) Arabidopsis nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. Plant Cell 17:3436–3450

Guo YF, Gan SS (2005) Leaf senescence: signals, execution, and regulation. Curr Top Dev Biol 71:83

Guo YF, Gan SS (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46:601–612

Hafsi M, Mechmeche W, Bouamama L, Djekoune A, Zaharieva M, Monneveux P (2000) Flag leaf senescence, as evaluated by numerical image analysis, and its relationship with yield under drought in durum wheat. J Agron Crop Sci 185:275–280

Ham B-K, Park J, Lee A-B, Kim M, Lee I-J, Kim K-J, Kwon C, Paek K-H (2006) Tobacco Tsip1, a DnaJ-type Zn finger protein, is recruited to and potentiates Tsi-mediated transcriptional activation. Plant Cell 18:2005–2020

Hanson J, Smeekens S (2009) Sugar perception and signaling – an update. Curr Opin Plant Biol 12:562–567

He Y, Fukushige H, Hildebrand DF, Gan S (2002) Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol 128:876–884

He P, Osaki M, Takebe M, Shinano T, Wasaki J (2005) Endogenous hormones and expression of senescence-related genes in different senescent types of maize. J Exp Bot 56:1117–1128

Hensel LL, Grbic V, Baumgarten DA, Bleecker AB (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. Plant Cell 5:553–564

Hilditch P, Thomas H, Rogers L (1986) Leaf senescence in a nonyellowing mutant of Festuca pratensis – photosynthesis and photosynthetic electron transport. Planta 167:146–151

Hörtensteiner S (2009) Stay-green regulates chlorophyll and chlorophyll-binding protein degradation during senescence. Trends Plant Sci 14:155–162

Humbeck K, Quast S, Krupinska K (1996) Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants. Plant Cell Environ 19:337–344

Huner NPA, Maxwell DP, Gray GR, Savitch LV, Krol M, Ivanov AG, Falk S (1996) Sensing environmental temperature change through imbalances between energy supply and energy consumption: redox state of photosystem II. Physiol Plant 98:358–364

Hung KT, Kao CH (2004) Hydrogen peroxide is necessary for abscisic acid-induced senescence of rice leaves. J Plant Physiol 161:1347–1357

Inaba T, Ito-Inaba Y (2010) Versatile roles of plastids in plant growth and development. Plant Cell Physiol 51:1847–1853

Innes P, Blackwell RD (1983) Some effects of leaf posture on the yield and water economy of winter wheat. J Agric Sci 101:367–376

Inze A, Vanderauwera S, Hoeberichts FA, Vandorpe M, Van Gaever T, Van Breusegem F (2012) A subcellular localization compendium of hydrogen peroxide-induced proteins. Plant Cell Environ 35:308–320

Iqbal M, Navabi A, Salmon DF, Yang RC, Spaner D (2007) Simultaneous selection for early maturity, increased grain yield and elevated grain protein content in spring wheat. Plant Breed 126:244–250

Isemer R, Mulisch M, Schäfer A, Kirchner S, Koop HU, Krupinska K (2012) Recombinant Whirly1 translocates from transplastomic chloroplasts to the nucleus. FEBS Lett 586:85–88

Ishida H, Yoshimoto K, Izuni M, Reisen D, Yano Y, Makino A, Ohsumi Y, Hanson M, Mae T (2008) Mobilization of Rubisco and stroma-localized fluorescent proteins of chloroplasts to the vacuole by an ATG gene-dependent autophagic process. Plant Physiol 148:142–155

Isidro J, Alvara F, Royo C, Villegas D, Miralles DJ, del Moral LFG (2011) Changes in duration of developmental phases of durum wheat caused by breeding in Spain and Italy during the 20th century and its impact on yield. Ann Bot 107:1355–1366

Izumi M, Ishida H (2011) The changes of leaf carbohydrate contents as a regulator of autophagic degradation of chloroplasts via rubisco-containing bodies during leaf senescence. Plant Signal Behav 6:685–687

Izumi M, Wada S, Makino A, Ishida H (2010) The autophagic degradation of chloroplasts via rubisco-containing bodies is specifically linked to leaf carbon status but not nitrogen status in Arabidopsis. Plant Physiol 154:1196–1209

Jing H-C, Hebeler R, Oeljeklaus S, Sitek B, Stühler K, Meyer H, Sturre M, Hille J, Warscheid B, Dijkwel P (2008) Early leaf senescence is associated with an altered cellular redox balance in Arabidopsis cpr5/old1 mutants. Plant Biol 10:85–89

Karpinski S, Gabrys H, Mateo A, Karpinska B, Mullineaux PM (2003) Light perception in plant disease defence signalling. Curr Opin Plant Biol 6:390–396

Kerchev PI, Pellny TK, Vivancos PD, Kiddle G, Hedden P, Driscoll S, Vanacker H, Verrier PJ, Hancock RD, Foyer CH (2011) The transcription factor ABI-4 is required for the ascorbic acid-dependent regulation of growth and regulation of jasmonate-dependent defense signaling pathways in Arabidopsis. Plant Cell 23:3319–3334

Kim C, Lee KP, Baruah A, Nater M, Gobel C, Feussner I, Apel K (2009) O-1(2)-mediated retrograde signaling during late embryogenesis predetermines plastid differentiation in seedlings by recruiting abscisic acid. Proc Natl Acad Sci U S A 106:9920–9924

Kingston-Smith AH, Thomas H, Foyer CH (1997) Chlorophyll a fluorescence, enzyme and antioxidant analysis provide evidence for the operation of alternative electron sinks during leaf senescence in a stay-green mutant of Festuca pratensis. Plant Cell Environ 20:1323–1337

Krupinska K, Humbeck K (2004) Photosynthesis and chloroplast breakdown (Review). In: Noóden LD (ed) Plant cell death processes. Elsevier, San Diego, CA, pp 169–188

Krupinska K, Mulisch M, Hollmann J, Tokarzc K, Zschiesche W, Kage H, Humbeck K, Bilger W (2012) An alternative strategy of dismantling of the chloroplasts during leaf senescence observed in a high yield variety of barley. Physiol Plant 144:189–200

Kura-Hotta M, Satoh K, Katoh S (1987) Relationship between photosynthesis and chlorophyll content during leaf senescence of rice seedlings. Plant Cell Physiol 28:1321–1329

Lee KP, Kim C, Landgraf F, Apel K (2007) EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of Arabidopsis thaliana. Proc Natl Acad Sci U S A 104:10270–10275

Lehto K, Tikkanen M, Hiriart JB, Paakkarinen V, Aro EM (2003) Depletion of the photosystem II core complex in mature tobacco leaves infected by the flavum strain of tobacco mosaic virus. Mol Plant Microbe Interact 16:1135–1144

Leister D (2005) Genomics-based dissection of the cross-talk of chloroplasts with the nucleus and mitochondria in Arabidopsis. Gene 354:110–116

Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. Annu Rev Plant Biol 58:115–136

Lu QT, Wen XG, Lu CM, Zhang QD, Kuang TY (2003) Photoinhibition and photoprotection in senescent leaves of field-grown wheat plants. Plant Physiol Biochem 41:749–754

Luo PG, Zhang HY, Shu K, Wu XH, Zhang HQ, Ren ZL (2009) The physiological genetic effects of 1BL/1RS translocated chromosome in "stay green" wheat cultivar CN17. Can J Plant Sci 89:1–10

Mateo A, Funck D, Mühlenbock P, Kular B, Mullineaux PM, Karpinski S (2006) Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. J Exp Bot 57:1795–1807

Miao Y, Laun T, Zimmermann P, Zentgraf U (2004) Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. Plant Mol Biol 55:853–867

Miersch I, Heise J, Zelmer I, Humbeck K (2000) Differential degradation of the photosynthetic apparatus during leaf senescence in barley (Hordeum vulgare L.). Plant Biol 2:618–623

Mizrahi Y, Blumenfe A, Richmond AE (1970) Abscisic acid and transpiration in leaves in relation to osmotic root stress. Plant Physiol 46:169–171

Morris K, Mackerness SA, Page T, John F, Murphy AM, Carr JP, Buchanan-Wollaston V (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. Plant J 23:677–685

Mühlenbock P, Szechynska-Hebda M, Plaszczyca M, Baudo M, Mullineaux PM, Parker JE, Karpinska B, Karpinski S (2008) Chloroplast signaling and LESION SIMULATING DISEASE1 regulate crosstalk between light acclimation and immunity in Arabidopsis. Plant Cell 20:2339–2356

Naruoka Y, Sherman JD, Lanning SP, Blake NK, Martin JM, Talbert LE (2012) Genetic analysis of green leaf duration in spring wheat. Crop Sci 52:99–109

Navabpour S, Morris K, Allen R, Harrison E, A-H-Mackerness S, Buchanan-Wollaston V (2003) Expression of senescence-enhanced genes in response to oxidative stress. J Exp Bot 54:2285–2292

Op den Camp R, Przybyla D, Ochsenbein C, Laloi C, Kim C, Danon A, Wagner D, Hideg E, Göbel C, Fueussner I, Nater M, Apel K (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. Plant Cell 15:2320–2332

Parry MAJ, Reynolds M, Salvucci ME, Raines C, Andralojc PJ, Zhu XG, Price GD, Condon AG, Furbank RT (2011) Raising yield potential of wheat. II. Increasing photosynthetic capacity and efficiency. J Exp Bot 62:453–467

Parthier B (1990) Jasmonates – hormonal regulators or stress factors in leaf senescence. J Plant Growth Regul 9:57–63

Pastori GM, del Rio LA (1994) An activated-oxygen-mediated role for peroxisomes in the mechanism of senescence of pea leaves. Planta 193:385–391

Pavet V, Olmos E, Kiddle G, Mowla S, Kumar S, Antoniw J, Alvarez ME, Foyer CH (2005) Ascorbic acid deficiency activates cell death and disease resistance responses in Arabidopsis. Plant Physiol 139:1291–1303

Pfannschmidt T (2010) Plastidial retrograde signalling – a true "plastid factor" or just metabolite signatures? Trends Plant Sci 15:427–435

Pfannschmidt T, Bräutigam K, Wagner R, Dietzel L, Schröter Y, Steiner S, Nykytenko A (2009) Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding. Ann Bot 103:599–607

Philosoph-Hadas S, Hadas E, Aharoni N (1993) Characterization and use in Elisa of a new monoclonal antibody for quantification of abscisic acid in senescing rice leaves. Plant Growth Regul 12:71–78

Pignocchi C, Foyer CH (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. Curr Opin Plant Biol 6:379–389

Pogson BJ, Woo NS, Förster B, Small ID (2008) Plastid signalling to the nucleus and beyond. Trends Plant Sci 13:602–609

Price J, Laxmi A, St Martin SK, Jang JC (2004) Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. Plant Cell 16:2128–2150

Prins A, van Heerden P, Olmos E, Kunert K, Foyer C (2008) Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. J Exp Bot 59:1935–1950

Pruzinska A, Anders I, Aubry S, Schenk N, Tapernoux-Luthi E, Muller T, Krautler B, Hortensteiner S (2007) In vivo participation of red chlorophyll catabolite reductase in chlorophyll breakdown. Plant Cell 19:369–387

Quiles MJ, Garcia C, Cuello J (1995) Differential-effects of abscisic-acid and methyl jasmonate on endoproteinases in senescing barley leaves. Plant Growth Regul 16:197–204

Reynolds MP, Pellegrineschi A, Skovmand B (2005) Sink-limitation to yield and biomass: a summary of some investigations in spring wheat. Ann Appl Biol 146:39–49

Rivero R, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc Natl Acad Sci U S A 104:19631–19636

Rolland F, Sheen J (2005) Sugar sensing and signalling networks in plants. Biochem Soc Trans 33:269–271

Rosenwasser S, Rot I, Sollner E, Meyer AJ, Smith Y, Fluhr R, Leviatan N, Friedman H (2011) Organelles contribute differentially to reactive oxygen-species-related events during extended darkness. Plant Physiol 156:185–201

Rumeau D, Peltier G, Cournac L (2007) Chlororespiration and cyclic electron flow around PSI during photosynthesis and plant stress response. Plant Cell Environ 30:1041–1051

Schurmann P, Buchanan BB (2008) The ferredoxin/thioredoxin system of oxygenic photosynthesis. Antioxid Redox Signal 10:1235–1273

Schippers JHM, Jing H-C, Hille J, Dijkwel PP (2007) Developmental and hormonal control of leaf senescence. In: Gan S (ed) Senescence processes in plants. Blackwell, Oxford, pp 145–170

Schwacke R, Fischer K, Ketelsen B, Krupinska K, Krause K (2007) Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice. Mol Genet Genome 277:631–646

Seiler C, Harshavardhan VT, Rajesh K, Reddy PS, Strickert M, Rolletschek H, Scholz U, Wobus U, Sreenivasulu N (2011) ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. J Exp Bot 62:2615–2632

Shang Y, Yan L, Liu Z-Q, Cao Z, Mei C, Xin Q, Wu F-Q, Wang X-F, Du S-Y, Jiang T, Zhang X-F, Zhao R, Sun H-L, Liu R, Yu Y-T, Zhang D-P (2010) The Mg-Chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. Plant Cell 22:1909–1935

Soitamo AJ, Piippo M, Allahverdiyeva Y, Battchikova N, Aro EM (2008) Light has a specific role in modulating Arabidopsis gene expression at low temperature. BMC Plant Biol 8:13

Spano G, Di Fonzo N, Perrotta C, Platani C, Ronga G, Lawlor DW, Napier JA, Shewry PR (2003) Physiological characterization of 'stay green' mutants in durum wheat. J Exp Bot 54:1415–1420

Strawn MA, Marr SK, Inoue K, Inada N, Zubieta C, Wildermuth MC (2007) Arabidopsis isochorismate synthase functional in pathogen-induced salicylate biosynthesis exhibits properties consistent with a role in diverse stress responses. J Biol Chem 282:5919–5933

Sun XW, Feng PQ, Xu XM, Guo HL, Ma JF, Chi W, Lin RC, Lu CM, Zhang LX (2011) A chloroplast envelope-bound PHD transcription factor mediates chloroplast signals to the nucleus. Nat Commun 2:477

Tang YL, Wen XG, Lu CM (2005) Differential changes in degradation of chlorophyll-protein complexes of photosystem I and photosystem II during flag leaf senescence of rice. Plant Physiol Biochem 43:193–201

Thomas H, Stoddart J (1980) Leaf senescence. Annu Rev Plant Physiol 31:83–111

Tollenaar M, Lee EA (2006) Dissection of physiological processes underlying grain yield in maize by examining genetic improvement and heterosis. Maydica 51:399–408

Uauy C, Brevis J, Dubcovsky J (2006) The high grain protein content gene Gpc-B1 accelerates senescence and has pleiotropic effects on protein content in wheat. J Exp Bot 57:2785–2794

Vadez V, Deshpande SP, Kholova J, Hammer GL, Borrell AK, Talwar HS, Hash CT (2011a) Stay-green quantitative trait loci's effects on water extraction, transpiration efficiency and seed yield depend on recipient parent background. Funct Plant Biol 38:553–566

Vadez V, Krishnamurthy L, Hash CT, Upadhyaya HD, Borrell AK (2011b) Yield, transpiration efficiency, and water-use variations and their interrelationships in the sorghum reference collection. Crop Pasture Sci 62:645–655

Vaezi B, Bavei V, Shiran B (2010) Screening of barley genotypes for drought tolerance by agro-physiological traits in field condition. Afr J Agric Res 5:881–892

Vanacker H, Sandalio LM, Jimenez A, Palma JM, Corpas FJ, Meseguer V, Gomez M, Sevilla F, Leterrier M, Foyer CH, del Rio LA (2006) Roles for redox regulation in leaf senescence of pea plants grown on different sources of nitrogen nutrition. J Exp Bot 57:1735–1745

Wada S, Ishida H, Izumi M, Yoshimoto K, Ohsumi Y, Mae T, Makino A (2009) Autophagy plays a role in chloroplast degradation during senescence in individually darkened leaves. Plant Physiol 149:885–893

Wagner R, Pfannschmidt T (2006) Eukaryotic transcription factors in plastids – bioinformatic assessment and implications for the evolution of gene expression machineries in plants. Gene 381:62–70

Wagner D, Przybyla D, Op den Camp R, Kim C, Landgraf F, Lee K, Würsch M, Laloi C, Nater M, Hideg E, Aple K (2004) The genetic basis of singlet oxygen-induced stress response of Arabidopsis thaliana. Science 306:1183–1185

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414:562–565

Wind J, Smeekens S, Hanson J (2010) Sucrose: metabolite and signaling molecule. Phytochem 71:1610–1614

Wingler A, Delatte TL, O'Hara LE, Primavesi LF, Jhurreea D, Paul MJ, Schluepmann H (2012) Trehalose 6-phosphate is required for the onset of leaf senescence associated with high carbon availability. Plant Physiol 158:1241–1251

Wolf FT (1956) Changes in chlorophyll-a and chlorophyll-b in autumn leaves. Am J Bot 43:714–718

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG (2001) ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. Plant Cell 13:1779–1790

Woo HR, Goh CH, Park JH, Teyssendier de la Serva B, Kim JH, Paark VI, Nam HG (2002) Extended leaf longevity in the ore4-1 mutant of Arabidopsis with a reduced expression of a plastid ribosomal protein gene. Plant J 31(3):331–340

Woo HR, Kim JH, Nam HG, Lim PO (2004) The delayed leaf senescence mutants of Arabidopsis, ore1, ore3, and ore9 are tolerant to oxidative stress. Plant Cell Physiol 45:923–932

Wu A, Allu AD, Garapati P, Siddiqui H, Dortay H, Zanor MI, Asensi-Fabado MA, Munné-Bosch S, Antonio C, Tohge T, Fernie AR, Kaufmann K, Xue GP, Mueller-Roeber B, Balazadeh S (2012) JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis. Plant Cell 24:482–506

Yang JC, Zhang JH, Wang ZQ, Zhu QS, Liu LJ (2003) Involvement of abscisic acid and cytokinins in the senescence and remobilization of carbon reserves in wheat subjected to water stress during grain filling. Plant Cell Environ 26:1621–1631

Zapata JM, Guéra A, Esteban-Carrasco A, Martín M, Sabater B (2005) Chloroplasts regulate leaf senescence: delayed senescence in transgenic ndhF-defective tobacco. Cell Death Differ 12:1277–1284

Zhang YH, Primavesi LF, Jhurreea D, Andralojc PJ, Mitchell RAC, Powers SJ, Schluepmann H, Delatte T, Wingler A, Paul MJ (2009) Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. Plant Physiol 149:1860–1871

Zhu XG, Long SP, Ort DR (2010) Improving photosynthetic efficiency for greater yield. Annu Rev Plant Biol 61:235–261

Zimmermann P, Zentgraf U (2005) The correlation between oxidative stress and leaf senescence during plant development. Cell Mol Biol Lett 10:515–534

Chapter 8
Grain Development

Winfriede Weschke and Hans Weber

8.1 Introduction

Millions of years of plant evolution adapted seed composition towards the demand of the germinating embryo. Optimising seed composition to the needs of humans or livestock has been a task for conventional plant breeding over centuries, and nowadays seeds of crop plants are the foremost material for feed and food production. Nevertheless, there is ample space for further improvement of seed yield and composition. Especially, yield increase and stability under changing environmental conditions are the major tasks for plant breeders in the future. However, molecular biology, plant physiology and our knowledge of seed development can also greatly contribute to improve and stabilise seed yield.

Targeted changes of cereal crops require knowledge about the major factors regulating grain development. Modern *omics* technologies provide useful tools for in-depth analysis of molecular processes and identification of such factors. But these insights have to be combined with detailed structural knowledge to understand the network of the temporally nested and interconnected development of the different tissues. Laser-based tissue separation and subsequent transcriptome and metabolite analyses at the micro-scale have been proved to be suitable tools to uncover the network of molecular communication between supplying and receiving regions within developing grains.

Grain development is arranged in defined developmental stages and involves communication between maternal and filial grain tissues and sink-source interactions. The uptake, partitioning, mobilisation and storage of assimilates and nutrients are processes that clearly impact on grain structures during development and at maturity, influencing grain quality and yield. This chapter describes the

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development and structure of the barley grain. This knowledge can be used as a starting point to define approaches in order to improve grain yield and grain composition.

8.2 The Stages of Barley Grain Development

During the generative phase of plant development, gynoecium and androecium are generated, which are the source of the egg cell and pollen grains, respectively. After pollination, one of the two sperm cells released by the pollen tube enters the egg cell to produce the diploid zygote. The other sperm cell combines with the diploid nucleus of the central cell resulting in the primary triploid endosperm nucleus. This so-called double fertilisation initiates seed development. Except for their differences in ploidy, the two fertilisation products are genetically alike, but they develop into completely different structures. The zygote generates the embryo, the fertilised nucleus of the endosperm mother cell forms the endosperm. The latter tissue is persistent in cereals and is highly specialised for storage product accumulation.

After fertilisation, barley grains start to develop and enter the pre-storage phase when cell division and elongation takes place (Fig. 8.1a). During this phase, growth of the young grain is controlled by the maternal tissues, which consist mainly of the pericarp but contain also the chlorenchyma layer, a chlorophyll-containing tissue adjacent to the integuments surrounding endosperm syncytium and vacuole (Fig. 8.1c). During the pre-storage phase, lateral vascular bundles supply assimilates to the pericarp for growth and storage product accumulation. Thus, the pericarp represents the first sink of the developing grain. Cellularisation of the endosperm starts 3 days after flowering (DAF) in front of the main vascular bundle and is finished about 5 DAF. Further development of the filial part of the grain occurs in parallel with differentiation of the transfer tissues at the maternal-filial boundary consisting of the maternal nucellar projection (NP) and the filial endosperm transfer cells (ETCs). At 10 DAF, caryopsis elongation is finished, and the functional transfer tissues deliver nutrients and assimilates to the developing endosperm, assuring a high flux of assimilates into storage product biosynthesis. From 10 DAF onwards, sink strength of the endosperm is established resulting in massive accumulation of starch and storage proteins in both starchy endosperm and aleurone, leading to expansion of the caryopsis which is clearly visible from 14 DAF onwards (Fig. 8.1b).

The maternal grain tissues, especially the pericarp, represent the first sink of the developing grain (Radchuk et al. 2009; Sreenivasulu et al. 2010a; Weschke et al. 2003). During further development, the maternal grain part is degraded in favour of the growing endosperm. Structural 3-D models of developing grains generated from segmented histological sections allow us to visualise and calculate changes of internal tissue volumes and their percentages within the whole caryopsis (Gubatz et al. 2007). Volumes of pericarp and endosperm dramatically decrease and increase respectively, especially between 7 and 10 DAF (Table 8.1).

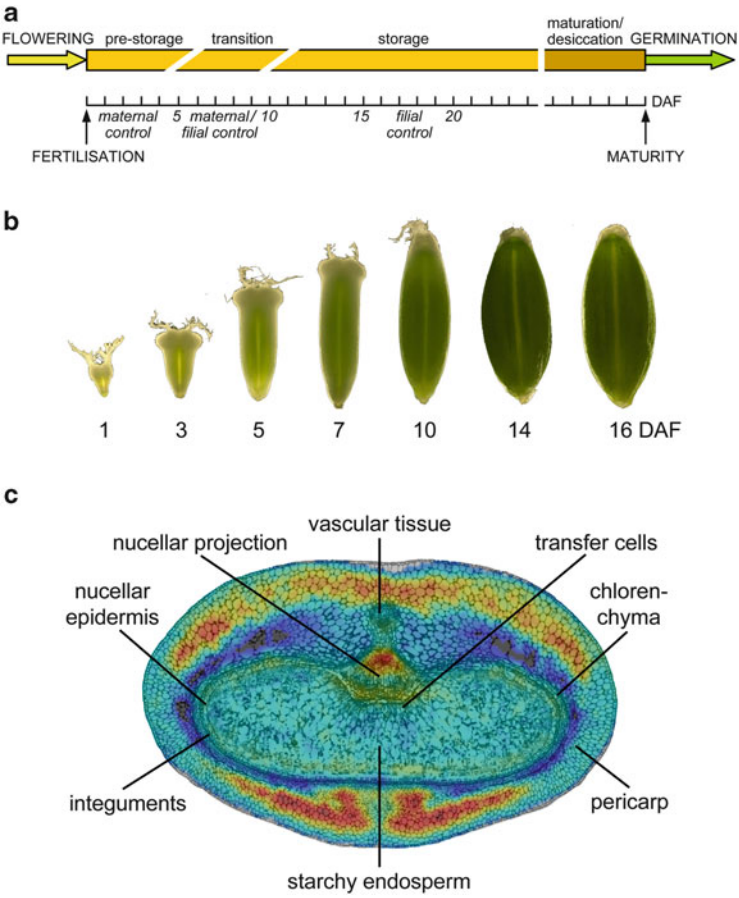

Fig. 8.1 Phases of barley grain development and structure of a developing grain at 7 days after flowering (DAF). (**a**) Schematic drawing representing the phases of grain development. (**b**) Developing barley grains between 1 and 16 DAF. Photographs are taken after removing the glumes. (**c**) Median-transverse section of a developing grain at 7 DAF showing an overlay of microscopic and NMR slices. The NMR signals represent the in vivo proton distribution (*red*, high proton density; *blue*, low proton density). Position and name of distinct caryopsis tissues are shown

At 10 DAF, maternal and filial tissues account for nearly the same percentages of the caryopsis. Apparently, this equal volume ratio of maternal and filial organs is concomitant with the beginning of the filial control of seed development. The strong increase of the endosperm volume between 3 and 5 DAF (Table 8.1) occurs by rapidly proceeding endosperm cellularisation. High volume increase between 7 and 10 DAF indicates the beginning of massive storage product accumulation.

Table 8.1 Percentages of volumes of five tissues of developing barley grains and caryopses fresh weight at 3, 5, 7, 10 and 14 days after flowering (DAF)

	Vascular bundle	Nucellar projection	Starchy endosperm	Pericarp	Endosperm transfer cells	Caryopsis weight (mg)
3 DAF	0.71 ± 0.12	1.22 ± 0.15	2.44 ± 1.2	95.63 ± 1.28	0	5.53 ± 0.93
5 DAF	0.66 ± 0.02	1.69 ± 0.19	8.29 ± 1.59	88.89 ± 1.98	0.49 ± 0.23	10.5 ± 0.82
7 DAF	0.74 ± 0.06	1.77 ± 0.15	14.36 ± 5.08	82.04 ± 5.35	1.07 ± 0.19	14.5 ± 2.93
10 DAF	0.68 ± 0.04	2.62 ± 0.76	41.87 ± 1.72	53.43 ± 1.55	1.40 ± 0.36	32.18 ± 2.23
14 DAF	0.37 ± 0.03	0.87 ± 0.12	63.18 ± 2.49	30.81 ± 2.31	0.58 ± 0.14	62.32 ± 2.31

The values ± standard deviation are calculated from segmented 3-D models of five individual grains from each developmental stage (F. Bollenbeck, W. Weschke, unpublished results)

8.3 The Maternal Tissues

8.3.1 Growing Processes and Cellular Disintegration in the Pericarp

In the young developing grain, the pericarp consists of the outer epidermis, several layers of parenchymatic cells, a three-cell-layered chlorenchyma and, up to about 4 DAF, the inner integument (Krauß 1933; Cochrane and Duffus 1983; Gubatz and Shewry 2011). The main adaxial bundle of the developing grain runs along the crease up to the distal end of the placenta-chalazal zone. Nutrients are supplied to the young grain through the main adaxial and the lateral bundles. Remobilisation processes in the style may supply metabolites especially to the upper parts of the young pericarp as indicated by the gradual expression of cell-wall-bound invertase HvCWINV2 at 2 DAF (Wobus et al. 2004).

Assimilate uptake results in reserve accumulation around the lateral vascular bundles and, later on, in close vicinity to the upper part of the NP (Radchuk et al. 2009; Weschke et al. 2000). Remobilisation of storage compounds and cellular disintegration is initiated during pericarp maturation, initially in regions of the style and then in regions surrounding the vascular bundles (Radchuk et al. 2009). Accordingly, HvVPE4, a specific vacuolar processing enzyme associated with programmed cell death, is expressed in pericarp parenchyma cells. Together with DNA fragmentation within the nuclei of the same region, HvVPE4-gene expression points to a specific form of programmed cell death within the pericarp (Radchuk et al. 2011).

In summary, the pericarp represents the first sink of the developing grain, which might provide nutrients for the endosperm. During the late stages of grain development, the pericarp builds up a protective hull. The regulatory role of the persisting chlorenchyma is poorly understood. This tissue might be photosynthetically active providing oxygen released by photosynthesis to improve respiration and thereby the energy state of the developing endosperm (Rolletschek et al. 2004). A possible role of the chlorenchyma in regulating the abscisic acid (ABA) content of the developing grain is suggested from the analysis of the maternal-affect endosperm mutant *seg8* (Sreenivasulu et al. 2010b).

8.3.2 Nucellus Degeneration and Nucellar Projection

The nucellus, a tissue of maternal origin, is located between the integuments and the embryo sac. Already at anthesis, starch granules are detectable in the nucellar cells. The starch is rapidly degraded, as suggested from accumulation of transcripts for α-amylase AMY4 in the nucellus at 2 DAF (Radchuk et al. 2009). The nutrients released by the degradation of the nucellus may be directly taken up by transporters located in the plasma membrane of the endosperm mother cell (Weschke

et al. 2003). Thereby, developmental processes in the syncytial endosperm are supported. Whereas the lateral and dorsal parts of the nucellus disintegrate by fine-tuned developmentally regulated disintegration processes, the ventral zone of the nucellus differentiates into the NP, a cup-like structure that extends towards the endosperm. The functional NP at 8 DAF consists of different regions, a part facing the main vascular bundle with actively dividing cells, a middle part with transfer cell-like structures and a part adjacent to the endosperm cavity with cells undergoing autolysis. The regulatory network which drives the complete differentiation process is unknown. However, *Jekyll* encoding a small cysteine-rich protein (Radchuk et al. 2006) plays a decisive role in the differentiation of the NP in barley. Jekyll is involved in the regulation of programmed cell death necessary for proper differentiation. Downregulation of *Jekyll* impairs assimilate flow to the endosperm and leads to the formation of irregular and small-sized grains at maturity. Tissue-specific transcriptome analysis of the NP revealed a role for gibberellin (GA) for establishment and maintenance of the differentiation gradient (Thiel et al. 2008). Amino acid analysis within the NP suggests a metabolic interface of amino acid reconversions in order to optimise amino acid supply to the developing endosperm (Thiel et al. 2009).

8.3.3 Maternal Influences on Seed Size

Seed size is a major determinant of the yield parameter seed weight. Cell elongation rather than cell division regulates endosperm growth and affects seed size in *Arabidopsis*. This was shown by analysis of the mutant *transparent testa glabra2* (*ttg2*) in which cell elongation in the integument is prevented. The combination of the TTG2 defective seed integument with reduced endosperm size by *iku* and *miniseed* mutations identified integument cell elongation and endosperm growth as the primary regulators of seed size (Garcia et al. 2005; Luo et al. 2005). On the other hand, increasing the number of seed integument cells causes a symmetrical increase in endosperm growth (Schruff et al. 2006). These results indicate that the capacity of the seed integuments regulates endosperm growth by genetic determinants expressed in the maternal sporophyte but also show the central role taken by the endosperm after fertilisation. The mechanistic nature of the communication between the endosperm and integuments remains unknown (Berger et al. 2006). A possible factor could be biophysical forces as the growing endosperm exerts mechanical pressure on the integument cells (Garcia et al. 2005; Haughn and Chaudhury 2005).

In *Arabidopsis*, analysis of natural allelic variation at seed size loci showed that variation in cell number was mainly controlled by maternal factors whereas non-maternal allelic variation mostly affected cell size (Alonso-Blanco et al. 1999). In crop plants, grain yield is controlled by quantitative trait loci (QTLs). Some of the genes underlying major QTLs for grain length, width and weight have been cloned and characterised from rice (Fan et al. 2006; Shomura

et al. 2008; Song et al. 2007). *GW2*, a QTL for rice grain width and weight, encodes a previously unknown RING-type E3 ubiquitin ligase that negatively regulates cell division. Reduction or loss of function of *GW2* leads to increased grain weight. *GW2* also has pleiotropic effects: it increases the cell number and therefore the width of the spikelet hull and also increases endosperm size, but mainly by cell expansion. The authors conclude that the larger cell size of the endosperm and heavier grain may result from faster rates of accumulation of dry matter. This may be caused by the larger spikelet hull providing a greater area of contact of the endosperm with the seed coat. Hence, enhanced endosperm size might be an indirect effect originating from the effect of *GW2* on sporophytic tissues (Song et al. 2007). Similar processes might explain the influence of QTL *qSW5* involved in determination of grain width in rice (Shomura et al. 2008). Deletion in *qSW5* significantly increased seed size owing to an increase in cell number in the outer glume of the rice flower. Reduction or loss of function of both *GW2* and *qSW5* results in increased source capacity that influences sink strength and leads to higher grain weight. As suggested for *Arabidopsis* (Alonso-Blanco et al. 1999), variation in cell size in the rice endosperm may also be controlled by resource allocation between the mother plant and the seed, whereas variation in cell number may be controlled maternally. Carpel weight, grain length and the relationship between fresh and dry weight of the grain are positively associated with the final grain weight of wheat (Hasan et al. 2011).

8.4 The Filial Grain Part

8.4.1 The Developing Endosperm

8.4.1.1 Endosperm Transfer Cells

Cellularisation of the syncytial endosperm begins in opposite to the developing NP at about 3 DAF, as indicated by the localised expression of END1 mRNA, which encodes a gene product with unknown function (Doan et al. 1996; Drea et al. 2005; Olsen 2004). From this region, cellularisation spreads to the outer parts of the endosperm and is accomplished at 5 DAF. Flange-like secondary wall ingrowths appear between 5 and 7 DAF within the three outermost endosperm cell layers in opposite to the NP (Thiel et al. 2012a) indicating initiation of transfer cell differentiation.

Transfer cells occur in many plants and are characterised by secondary wall ingrowths and wall thickening which amplify the membrane surface and thereby effectively increase assimilate flux (Bonnemain et al. 1991; Gunning 1977; Thompson et al. 2001). The timing and extent of wall modifications characterising transfer cells are tightly regulated by the availability of solutes and/or by stress (Offler et al. 2002). Plant transfer cells can be subdivided into two categories based on the morphology of cell walls, flange-like and reticulate types (McCurdy et al. 2008).

Maize basal ETCs (Davis et al. 1990) and wheat ETCs (Zheng and Wang 2011) are of the flange-like type. They are organised as parallel, branched, ridge-like depositions of wall material. Bundles of microtubules play an important role during their deposition (Talbot et al. 2007). Auxin (Doblin et al. 2009), glucose (Andriunas et al. 2011), ethylene (Zhou et al. 2010) and reactive oxygen species (Andriunas et al. 2012) have been proposed to initiate signal cascades that lead to the induction of ingrowths from the wall. Accordingly, AP2/EREBP-like transcription factors (APETALA2/ethylene-responsive element-binding protein) and ethylene metabolism and signal transduction are found to be transcriptionally activated in barley ETCs (Thiel et al. 2008). Transcriptome analyses of developing ETCs at the microscale indicated that ethylene-signalling pathways initiate ETC morphology in barley between 7 and 10 DAF (Thiel et al. 2012a). After 10 DAF, the cells appear to undergo further differentiation. The activation of storage and stress-related processes indicate metabolic reprogramming of the cells, which is possibly initiated by ABA.

Roles for both ABA and ethylene in the early differentiation of ETCs have also been indicated by large-scale pyrosequencing of cellularising and differentiating barley ETCs (Thiel et al. 2012b). The ETC transcriptome showed a high abundance of elements of the two-component signalling (TCS) system, a multistep phosphorelay that influences a variety of processes in plants, usually by hormonal regulation (Schaller et al. 2008). A gene encoding a specific membrane-bound kinase, *HvHK1*, was found to be highly expressed between 3 and 5 DAF. The amino acid sequence is similar to AHK1 from *Arabidopsis* initially identified as a plant osmosensor, which positively affects ABA signalling and enhances ABA biosynthesis in vegetative and seed tissues under osmotic stress. Together with evidence for ABA-dependent transcriptional regulation of the HvHK1 phosphorelay, the authors suggest a crosstalk of ABA and TCS elements expressed in ETCs during the switch from the syncytial stage to cellularisation. Confirming the results from transcriptome analyses of microdissected ETCs (Thiel et al. 2012a), ethylene control of ETC differentiation at 5 and 7 DAF was further proposed. Two different types of phosphorelays either being highly ETC specific or spreading throughout the whole grain were suggested to mediate ethylene signals via ethylene-responsive transcription factors (Thiel et al. 2012b).

8.4.1.2 Establishment of Endosperm Sink Strength

When endosperm cellularisation and endosperm transfer cell differentiation are completed, endoreduplication and storage product synthesis are initiated. The beginning of grain filling and the establishment of seed sink strength is accompanied by massive transcriptional reprogramming (Sreenivasulu et al. 2004). Thereby, the endosperm switches from cell proliferation into the storage mode accompanied by upregulated expression of genes related to starch and storage protein biosynthesis. Whereas cell proliferation is predominantly under maternal control, storage product accumulation during the filling phase is controlled by the sink

strength of the filial organs. Thereby, assimilate availability and metabolic control play a major role in modulating signals that control grain filling.

Seeds take up sucrose and amino acids. While seed storage protein biosynthesis in general depends on nitrogen, sucrose also has specific functions as a transport and nutrient sugar and as a signal molecule (Koch 2004; Smeekens 2000). In the barley endosperm, the increase of sucrose levels at the onset of grain filling (Weschke et al. 2000) marks the switch from maternal to filial control of seed growth and is associated with maturation. In legumes and barley, sucrose induces the expression of genes associated with storage activities at the transcript level and increases the levels of enzymes such as sucrose synthase and ADP-glucose pyrophosphorylase (Weigelt et al. 2009; Weschke et al. 2000). The function of sucrose is tightly associated with that of ABA, SnRK1-like kinases and trehalose metabolism (Radchuk et al. 2010a, b; Sreenivasulu et al. 2006a; Thiel et al. 2008).

Cell-wall-bound invertases (CWINVs) are generally active in growing zones and expanding sink tissues. These invertases facilitate the unloading of assimilate by increasing the concentration gradient of sucrose (Weber et al. 1995). Several lines of evidence indicate that cell wall invertases play a critical role in establishing sink strength (Lara et al. 2004; Roitsch and González 2004; Zhang et al. 1996). CWINVs catalyse the unidirectional conversion of sucrose into glucose and fructose, which are imported into the sink tissue by hexose transporters (Roitsch et al. 2003). The presence of cell wall invertases is critical for the import of photoassimilates into developing seeds in maize (Miller and Chourey 1992), bean (Weber et al. 1997), tomato (Fridman et al. 2004) and barley (Weschke et al. 2003). CWINVs are expressed in the unloading area of the developing seed, where they cleave unloaded sucrose within the apoplastic space separating the maternal and filial tissues. Thereby a high-hexose environment is created at a time when mitotic activity occurs in the filial tissues. In beans, the role of CWINV in promoting mitotic activity is in accordance with the maternal control of embryo cell number (Weber et al. 1996). In maize, kernels of the CWINV-deficient *miniature 1* mutant show a phenotype of decreased seed size possibly caused by decreased apoplastic hexose contents and by altered phytohormone levels. This suggests that the effects of invertases on sugars in sink tissues may influence sink size and strength through the regulation of phytohormone levels (LeClere et al. 2008).

It is thought that cereal grains grow under saturated assimilate (source) supply (Borrás et al. 2003). Thus, it can be suggested that grain sink strength is a critical yield limiting factor at post-anthesis which can be improved by modulating the balance between the source and the sink (Reynolds et al. 2011). Sucrose is a key player in the regulatory network controlling seed filling and maturation (Weber et al. 2005). Because in seeds the filial tissue is apoplastically isolated from the maternal tissue, membrane-localised transport steps are necessary for sucrose import. Sucrose transporters are encoded by small gene families. The rate-limiting role of sucrose transporters has been demonstrated for rice (*Oryza sativa*). Thereby, suppression of *OsSUT1* reduces the starch content of the grain and leads to a wrinkled phenotype (Scofield et al. 2002). The sucrose transporter HvSUT1 from barley is preferentially expressed in ETCs of developing grains (Weschke

et al. 2000). Overexpression of HvSUT1 in transgenic wheat grains increased sucrose uptake capacity (Weichert et al. 2010). HvSUT1 overexpression also deregulates the metabolic status, which is shown by the upregulated expression of genes encoding positive and negative regulators related to sugar signalling and assimilate supply. The oscillatory pattern of gene expression highlights the capacity and great flexibility of developing cereal grains to adjust the storage metabolism in response to metabolic alterations. As a result, an increased flux of sucrose into metabolic processes is sensed, resulting in larger size of mature grains (Weichert et al. 2010).

8.4.1.3 Hormonal Influences on Endosperm Development

Mitotic activity is regulated by gibberellins (Swain et al. 1995) and cytokinin (Emery and Atkins 2006) and assimilates supply to the seed (Egli et al. 1989). During early endosperm development, high levels of cytokinins (CKs) may enhance sink strength and attract assimilates via the promotion of cell division and the metabolism of imported sucrose (Quesnelle and Emery 2007). Accordingly, CKs are involved in the control of grain sink strength in rice (Yang et al. 2002) and maize (Rijavec et al. 2009). Moreover, CKs are involved in the differentiation of amyloplasts and stimulate the expression of the gene for the small subunit of ADP-glucose pyrophosphorylase (AGP) (Miyazawa et al. 2002). This relationship is corroborated by transcript profiling and hormone measurements in the barley mutant *Risø 16* that shows decreased CK levels accompanied by the absence of AGP and reduced starch biosynthesis (Faix et al. 2012). It can be speculated that the decreased CK/ABA levels and the interplay between CK and ABA signalling might lead to a coordinated transcriptional downregulation of endosperm-specific gene expression. This may explain the decreased carbon flux into starch in the developing *Risø 16* endosperm (Faix et al. 2012).

The role of phytohormones, especially ABA, to regulate transcriptional networks during differentiation and maturation of endosperm and embryo of barley was reviewed by Sreenivasulu et al. (2010a). ABA levels are high during the early development of barley grains and during the storage product accumulation. It was suggested that the expression of genes for ABA biosynthesis correlates with the presence of the hormone (Seiler et al. 2011) with NCED being the key enzyme of ABA biosynthesis (Iuchi et al. 2001; Taylor et al. 2000). However, the ABA content of a specific tissue depends not only on biosynthesis. Transport, degradation, conjugation and feedback regulation also influence ABA concentration. In the recessive endosperm mutant *seg8* (Felker et al. 1985; Röder et al. 2006), altered ABA levels have effects on both endosperm cellularisation and grain filling (Sreenivasulu et al. 2010b). Compared to wild-type grains, the ABA levels were lower in *seg8* during the pre-storage phase but higher during the transition to storage accumulation. The endoploidy levels and amounts of ABA were inversely correlated in the developing endosperms of the mutant and wild-type lines, suggesting that ABA may affect cell cycle regulation. Effects on endoreduplication

in the endosperm and on grain filling were also observed in rice (Barrôco et al. 2006). Furthermore, it appears that endosperm cell division and, to a lesser extent, endoreduplication in early maize kernels are inhibited by exogenously applied ABA (Mambelli and Setter 1998; Myers et al. 1990).

ABA is also a major determinant of dormancy (Millar et al. 2006). The interactions of ABA and GA regulate the cell death programme in cereal aleurone cells (Guo and Ho 2008), and the balance between GA and ABA is a determining factor during the transition from embryogenesis to seed germination (Schoonheim et al. 2007, 2009). To promote seed germination, GAs are transported to the aleurone layer to antagonise the effect of the dormancy hormone ABA and to induce the expression of genes encoding a range of hydrolytic enzymes (Kaneko et al. 2003). The concerted action of GA and ABA regulates the expression level of α-amylases by modulating the relative amounts of repressor and activator complexes that bind to regulatory *cis*-elements in the promoter region (Zou et al. 2008).

8.4.1.4 Endopolyploidisation

Endopolyploidisation is often observed in highly differentiated organs such as starch-storing tissues (Chojecki et al. 1986) and is common in the cereal endosperm (Dermastia 2009). Its onset often correlates with cell expansion (Breuer et al. 2010). In general, ploidy levels in plant cells are positively correlated with cell size (Breuer et al. 2010) and higher transcriptional capacity (Larkins et al. 2001). However, endoreduplication is not strictly coupled with cell size and gene transcription (Nguyen et al. 2007). Endoreduplication follows a switch from the normal cell cycle to the endocycle in which mitosis is inhibited (Sugimoto-Shirasu and Roberts 2003). Cyclin-dependant kinases and their inhibitors regulate the progression from the G2 phase to mitosis (De Veylder et al. 2011; Sabelli and Larkins 2009b). A reduction in ploidy levels through manipulation of the kinase did not lead to expected decreases of starch contents in maize endosperm (Leiva-Neto et al. 2004) or of cell size in the tomato pericarp (Nafati et al. 2011). In the barley mutant *seg8*, altered expression of cell cycle-related marker genes was suggested to be responsible for the reduction in ploidy level (Sreenivasulu et al. 2010b).

The DNA content of barley grains increases about tenfold between 5 and 25 DAF, and a gradient of ploidy levels was observed reflected by increased nuclear size towards the centre of the endosperm (Giese 1991). 24 C nuclei were observed for endosperm cells in allohexaploid wheat indicating three steps of endoreduplication (Chojecki et al. 1986), and small amounts of 48 C nuclei are present in the barley endosperm (Sreenivasulu et al. 2010b). Non-stressed maize plants reached higher ploidy levels in the endosperm compared to heat-stressed plants (Bringezu et al. 2011).

In the endosperm of cereals, endoreduplication is associated with increased nuclear and cell sizes and with increased metabolic rates and cell differentiation (reviewed in Sabelli and Larkins 2009a). Endoreduplication in the cereal endosperm is also correlated with the biosynthetic capability of the tissue (Larkins

et al. 2001). The increase in the number of DNA templates could support higher transcription and translation rates (D'Amato 1984) and may lead to increased gene expression, greater protein synthesis and higher metabolic rates. The chromatin in endoreduplicated endosperm nuclei is loosely condensed, which may result in facilitated access for transcription factors, which leads to higher transcription rates (Sabelli 2012). In rice, the milky endosperm is characterised by hypomethylation, a state which correlates with the expression of a number of genes involved in starch and protein accumulation (Zemach et al. 2010). During endoreduplication, the ratio of cell volume to endosperm cell walls is kept to a minimum, whereas repeated cell division would result in many small cells (Kowles 2009). Because cell wall biosynthesis is strongly energy consuming, lower rates of wall biosynthesis could reduce the energy consumption and may allow a higher level of storage product synthesis.

8.4.1.5 The Embryo-Surrounding Region

The embryo-surrounding region (ESR) originally comprises a small region adjacent to the maize embryo, defined by the expression of *Esr* genes (Cosségal et al. 2007). In embryo-less kernels no ESR-specific gene expression is detected indicating that embryo-derived signals are required for correct gene expression (Opsahl-Ferstad et al. 1997). Like in maize, this region in wheat, barley and *Arabidopsis* is cytologically different from the remaining endosperm. Both morphology and gene expression specify a particular role for the ESR with a characteristic genetic programme. In barley, the endosperm adjacent to the embryo has a richer cytoplasm and higher nuclei density and shows earlier cellularisation compared to the remaining endosperm (Engell 1989; Brown et al. 1994). ESR cells always remain smaller, have less vacuoles and are rich in ribosomes, rough ER and dictyosomes with large secretion vesicles. During embryo maturation, the ESR in maize, barley and wheat becomes lysed and potentially absorbed by the embryo, which leaves a liquid-filled cavern between the embryo and endosperm. Several ESR-specific genes have been identified in maize and *Arabidopsis* allowing to deduce putative functions (Bate et al. 2004; Hehenberger et al. 2012; Baud et al. 2005; Cosségal et al. 2007; Tanaka et al. 2001; Yang et al. 2008). Although the exact function of the ESR remains obscure, it may include embryo nutrition and defence, definition of a boundary between endosperm and embryo and signalling between embryo and endosperm.

8.4.2 The Developing Embryo

Proper development of the embryo is most important for survival and performance of the next generation. Embryo development in cereals has been studied at the

morphological level for many years (Norstog 1974; Engell 1989; Smart and O'Brien 1983; Raghavan 1986), but knowledge at the molecular level is scarce.

72–80 h after pollination, the protoderm is formed in the 14-cell stage embryo. At 8 DAF, the outermost cell layer of the apical embryo starts to differentiate into the scutellum, which forms a shield-shaped structure by linear growth. Auxin signalling cascades and H^+-ATPases are involved in scutellar cell elongation in wheat (Rober-Kleber et al. 2003) and in the growing barley embryo (Matzk 1991). The source of auxin in the embryo is unclear. It may be imported from ESR and aleurone which are found to be enriched in auxin. At 10–12 DAF, dorsal epidermal cells appear smaller and begin to elongate perpendicular to the surface at 16 DAF (Merry 1941). These cells may differentiate into transfer cells involved in assimilate uptake.

In monocotyledons the scutellum represents the single cotyledon of the embryo. The function of the scutellum has been analysed in some detail only during germination (Edelman et al. 1959; Salmenkallio and Sopanen 1989; Waterworth et al. 2000). During germination, storage proteins in the barley endosperm are hydrolysed, and the products, small peptides and amino acids, are taken up by the scutellum (Salmenkallio and Sopanen 1989). In the barley scutellum, peptide transport activity appears 6–12 h after imbibition, before germination is visible, and increases rapidly to a maximum of 24 h after imbibition (Sopanen 1979). HvPTR1 has been identified as the barley scutellar peptide transporter within the plasma membrane (Waterworth et al. 2000). Carbohydrates are also absorbed by the scutellum as glucose and subsequently are converted to sucrose (Edelman et al. 1959). Whereas the function of the scutellum as an absorbing and transfer organ for compounds is reasonably described for germination, its role as storage organ during development is largely neglected.

After the embryo is differentiated, storage product accumulation is initiated at 12 DAF indicated by gene expression related to starch, storage globulins and oleosin biosynthesis. Embryo-specific gene expression related to ABA function and biosynthesis differs from those in the endosperm (Sreenivasulu et al. 2006b). Starch accumulates transiently in the embryo and becomes mobilised during maturation (Duffus and Cochran 1993). Lipids accumulate in scutellum and embryo axis during grain maturation (Neuberger et al. 2008). The barley embryo contains 30 % of the grain lipid reserves but accounts for only 3 % of the grain mass. Thus, the embryo strongly determines the lipid content of the whole grain and therefore its nutritional value. Efforts have been ventured to screen for genotypes with increased lipids, which would significantly improve the forage quality (Price and Parsons 1979). The barley embryo is also enriched in microelements such as Fe and Zn (Lombi et al. 2011). High concentrations are present within the vascular bundle of the scutellum and occur in globoids, complexed by phytate. Microelement storage is highly relevant adverting the so-called hidden hunger. However, mechanisms and capabilities for microelement uptake and storage within the embryo are currently under-investigated. In addition, there is not much known about the origin and transfer of precursors for storage product biosynthesis and micronutrients into the barley scutellum during development.

Conclusions

Barley grain development relies on the concerted action of signalling cascades, which are initiated by fertilisation. This first initiates development of maternal tissues during the pre-storage phase. Development of the maternal grain part is coordinated with cellularisation, differentiation and storage product accumulation in the filial grain part. The sequence of development indicates that the foremost developing tissue has supplying functions for the following one. The comprehensive molecular and biochemical analyses as discussed in this chapter point to an interplay between hormones and metabolites, which influences also endosperm endoreduplication.

The pre-storage phase of grain development is under maternal control. Thus, the number of nuclei within the syncytial endosperm and the cell number within the major storage organ should be determined maternally. Also grain length is mainly maternally controlled. This can be concluded from attenuated increase in grain length after the switch from maternal to filial control at 10 DAF. From 10 DAF onwards, massive accumulation of storage products occurs, which obviously influences both grain width and grain thickness. The process is under filial control. Grain length, width and thickness determine grain size, which together with grain number is yield determining.

NP and ETCs play a key role in supplying the developing endosperm. Laser-dissection-based microtechniques and *omics* analyses allowed deep insights into differentiation and transfer processes especially of these tissues. However, the current knowledge is at the basic level of science. Transfer into applied approaches has to be developed in the future.

The mechanisms that regulate endosperm-embryo interactions are largely unknown such as communication between the early embryo, maternal tissues and ESR. To a larger extent, this is true also for sink-source communication during embryo filling. In contrast to the starchy endosperm, the embryo accumulates high amounts of lipids and specific micronutrients. However, the "hidden hunger" cannot be mitigated by simply increasing embryo micronutrient content unless mainly white flour or polished rice is favoured by consumers; embryo and aleurone are removed during milling or grain polishing. Towards increasing seed sink strength, it is most important to analyse the processes that partition nutrients to the different parts of the caryopsis and to uncover mechanisms that allow accumulation of higher amounts of micronutrient within the starchy endosperm.

References

Alonso-Blanco C, Blankestijn-de Vries H, Hanhart CJ, Koornneef M (1999) Natural allelic variation at seed size loci in relation to other life history traits of *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 96:4710–4717

Andriunas FA, Zhang HM, Weber H, McCurdy DW, Offler CE, Patrick JW (2011) Glucose and ethylene signalling pathways converge to regulate trans-differentiation of epidermal transfer cells in Vicia narbonensis cotyledons. Plant J 68:987–998

Andriunas FA, Zhang HM, Xia X, Offler CE, McCurdy DW, Patrick JW (2012) Reactive oxygen species form part of a regulatory pathway initiating trans-differentiation of epidermal transfer cells in Vicia faba cotyledons. J Exp Bot 63:3617–3630

Borrás L, Slafer GA, Otegui ME (2003) Seed dry weight response to source–sink manipulations in wheat, maize and soybean: a quantitative reappraisal. Field Crops Res 86:131–146

Barrôco RM, Peres A, Droual A-M, De Veylder L, Nguyen LSL, De Wolf J, Mironov V, Peerbolte R, Beemster GTS, Inze D, Broekaert WF, Frankard V (2006) The cyclin-dependent kinase inhibitor Orysa; KRP1 plays an important role in seed development of rice. Plant Physiol 142:1053–1064

Bate NJ, Niu X, Wang Y, Reimann KS, Helentjaris TG (2004) An invertase inhibitor from maize localizes to the embryo surrounding region during early kernel development. Plant Physiol 134:246–254

Baud S, Wuillème S, Lemoine R, Kronenberger J, Caboche M, Lepiniec L, Rochat C (2005) The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in Arabidopsis. Plant J 43:824–836

Berger F, Grini PE, Schnittger A (2006) Endosperm: an integrator of seed growth and development. Curr Opin Plant Biol 9:664–670

Bonnemain JL, Borquin S, Renault S, Offler CE, Fisher DG (1991) Transfer cells: structure and physiology. In: Bonnemain L, Delrot S (eds) Recent advances in phloem transport and assimilate compartmentation, Quest editions Nantes France, pp 74–83

Breuer C, Ishida T, Sugimoto K (2010) Developmental control of endocycles and cell growth in plants. Curr Opin Plant Biol 13:654–660

Bringezu TGG, Sharbel TF, Weber WE (2011) Grain development and endoreduplication in maize and the impact of heat stress. Euphytica 182:363–376

Brown RC, Lemon BE, Olsen OA (1994) Endosperm development in barley: microtubule involvement in the morphogenetic pathway. Plant Cell 6:1241–1252

Chojecki AJS, Bayliss MW, Gale MD (1986) Cell production and DNA accumulation in the wheat endosperm, and their association with grain weight. Ann Bot 58:809–817

Cochrane MP, Duffus CM (1983) Observations on the development of the testa and pericarp in barley. In: Kruger JE, LaBerge DE (eds) Third international symposium on pre-harvest sprouting in cereals (Hecla Island, Canada, June 1982). Westview Press, Boulder, CO, pp 154–161

Cosségal M, Vernoud V, Depège N, Rogowski PM (2007) The embryo surrounding region. Plant cell monography. Springer, Berlin, pp 52–72

D'Amato F (1984) Role of polyploidy in reproductive organs and tissues. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin, pp 523–566

Davis RW, Smith JD, Cobb BG (1990) A light and electron microscope investigation of the transfer cell region of maize caryopses. Can J Bot 68:471–479

De Veylder L, Larkin CJ, Schnittger A (2011) Molecular control and function of endoreplication in development and physiology. Trends Plant Sci 16:624–634

Dermastia M (2009) Endoreduplication in cereal plants. In: Devane RT (ed) New plant physiology research. Botanical research and practices. Nova Science, Hauppauge, pp 177–202

Doan DNP, Linnestad C, Olsen O-A (1996) Isolation of molecular markers from the barley endosperm coenocyte and the surrounding nucellus cell layers. Plant Mol Biol 31:877–886

Doblin MS, Pettolino FA, Wilson SM, Campbell R, Burton RA, Fincher GB, Newbigin E, Bacic A (2009) A barley *cellulose synthase-like CSLH* gene mediates (1,3;1,4)-β-D-glucan synthesis in transgenic *Arabidopsis*. Proc Natl Acad Sci U S A 106:5996–6001

Drea S, Leader DJ, Arnold BC, Shaw P, Dolan L, Doonan JH (2005) Systematic spatial analysis of gene expression during wheat caryopsis development. Plant Cell 17:2172–2185

Duffus CM, Cochran MP (1993) Formation of the barley grain – morphology, physiology, and biochemistry. In: Macgregor AW, Bhatty RS (eds) Barley: chemistry and technology. AACC, St. Paul, MN, pp 31–72

Edelman J, Shibko SI, Keys AJ (1959) The role of the scutellum of cereal seedlings in the synthesis and transport of sucrose. J Exp Bot 10:178–189

Egli DB, Ramseur EL, Zhenwen Y, Sullivan CH (1989) Source-sink alterations affect the number of cells in soybean cotyledons. Crop Sci 29:732–735

Emery N, Atkins CA (2006) Cytokinins and seed development. In: Basra AS (ed) Handbook of seed science and technology. Haworth Press (Food Products Press), Binghamton, NY, pp 63–93

Engell K (1989) Embryology of barley: time course and analysis of controlled fertilization and early embryo formation based on serial sections. Nordic J Bot 9:265–280

Faix B, Radchuk V, Nerlich A, Hümmer C, Radchuk R, Emery RJN, Keller H, Götz K-P, Weschke W, Geigenberger P, Weber H (2012) Barley grains, deficient in cytosolic small subunit of ADP-glucose pyrophosphorylase, reveal coordinate adjustment of C:N metabolism mediated by an overlapping metabolic-hormonal control. Plant J 69:1077–1093

Fan CH, Xing YZ, Mao HL, Lu TT, Han B, Xu CG, Li XH, Zhang QF (2006) *GS3*, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. Theor Appl Genet 112:1164–1171

Felker FC, Peterson DM, Nelson OE (1985) Anatomy of immature grains of eight maternal effect shrunken endosperm barley mutants. Am J Bot 72:248–256

Fridman E, Carrari F, Liu Y-S, Fernie AR, Zamir D (2004) Zooming in on a quantitative trait for tomato yield using interspecific introgressions. Science 305:1786–1789

Garcia D, Fitz Gerald JN, Berger F (2005) Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in Arabidopsis. Plant Cell 17:52–60

Giese H (1991) Replication of DNA during barley endosperm development. Can J Bot 70:313–318

Gubatz S, Shewry PR (2011) The development, structure, and composition of the barley grain. In: Ullrich SE (ed) Barley: production, improvement, and uses. Wiley-Blackwell, Chichester

Gubatz S, Dercksen VJ, Brüß C, Weschke W, Wobus U (2007) Analysis of barley (Hordeum vulgare) grain development using three-dimensional digital models. Plant J 52:779–790

Gunning BES (1977) Transfer cells and their role in transport of solutes in plants. Sci Prog 64: 539–568

Guo W-J, Ho T-HD (2008) An abscisic acid-induced protein, HVA22, inhibits gibberellin-mediated programmed cell death in cereal aleurone cells. Plant Physiol 147:1710–1722

Hasan AK, Herrera J, Lizana C, Calderini DF (2011) Carpel weight, grain length and stabilized grain water content are physiological drivers of grain weight determination of wheat. Field Crops Res 123:241–247

Haughn G, Chaudhury A (2005) Genetic analysis of seed coat development in *Arabidopsis*. Trends Plant Sci 10:472–477

Hehenberger E, Kradolfer D, Köhler C (2012) Endosperm cellularization defines an important developmental transition for embryo development. Development 139:2031–2039

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. Plant J 27:325–333

Kaneko M, Itoh H, Inukai Y, Sakamoto T, Ueguchi-Tanaka M, Ashikari M, Matsuoka M (2003) Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? Plant J 35: 104–115

Koch K (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opin Plant Biol 7:235–246
Kowles RV (2009) The importance of DNA endoreduplication in the developing endosperm of maize. Maydica 54:387–399
Krauß L (1933) Entwicklungsgeschichte der Früchte von Hordeum, Triticum, Bromus und Poa mit besonderer Berücksichtigung ihrer Samenschalen. Jahrbücher für wissenschaftliche Botanik 77:733–808
Lara MEB, Garcia M-CG, Fatima T, Ehneß R, Lee TK, Proels R, Tanner W, Roitsch T (2004) Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. Plant Cell 16:1276–1287
Larkins BA, Dilkes BD, Dante RA, Coelho CM, Woo Y-M, Liu Y (2001) Investigating the hows and whys of DNA endoreduplication. J Exp Bot 52:183–192
LeClere S, Schmelz EA, Chourey PS (2008) Cell wall invertase-deficient *miniature1* kernels have altered phytohormone levels. Phytochemistry 69:692–699
Leiva-Neto JT, Grafi G, Sabelli PA, Dante RA, Woo YM, Maddock S, Gordon-Kamm WJ, Larkins BA (2004) A dominant negative mutant of cyclin-dependent kinase A reduces endoreduplication but not cell size or gene expression in maize endosperm. Plant Cell 16:1854–1869
Lombi E, Smith E, Hansen TH, Paterson D, de Jonge MD, Howard DL, Persson DP, Husted S, Ryan C, Schjoerring JK (2011) Megapixel imaging of (micro)nutrients in mature barley grains. J Exp Bot 62:273–282
Luo M, Dennis ES, Berger F, Peacock WJ, Chaudhury A (2005) *MINISEED3* (*MINI3*), a *WRKY* family gene, and *HAIKU2* (*IKU2*), a leucine-rich repeat (*LRR*) *KINASE* gene, are regulators of seed size in *Arabidopsis*. Proc Natl Acad Sci U S A 102:17531–17536
Mambelli S, Setter TL (1998) Inhibition of maize endosperm cell division and endoreduplication by exogenously applied abscisic acid. Physiol Plant 104:266–272
Matzk F (1991) A novel approach to differentiated embryos in the absence of endosperm. Sex Plant Reprod 4:88–94
McCurdy DW, Patrick JW, Offler CE (2008) Wall ingrowth formation in transfer cells: novel examples of localized wall depositions in plant cells. Curr Opin Plant Biol 11:653–661
Merry J (1941) Studies of the embryo of barley. Bulletin of the Torrey Botanical Club, Lancester Business Pr 1870. ISSN 0040-9618
Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F (2006) Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8′-hydroxylase. Plant J 45:942–954
Miller ME, Chourey PS (1992) The maize invertase-deficient *miniature-1* seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell 4:297–305
Miyazawa Y, Kato H, Muranaka T, Yoshida S (2002) Amyloplast formation in cultured tobacco BY-2 cells requires a high cytokinin content. Plant Cell Physiol 43:1534–1541
Myers PN, Setter TL, Madison JT, Thompson JF (1990) Abscisic Acid inhibition of endosperm cell division in cultured maize kernels. Plant Physiol 94:1330–1336
Nafati M, Cheniclet C, Hernould M, Do PT, Fernie AR, Chevalier C, Gévaudant F (2011) The specific overexpression of a cyclin-dependent kinase inhibitor in tomato fruit mesocarp cells uncouples endoreduplication and cell growth. Plant J 65:543–556
Neuberger T, Sreenivasulu N, Rokitta M, Rolletschek H, Göbel C, Rutten T, Radchuk V, Feussner I, Wobus U, Jakob P, Webb A, Borisjuk L (2008) Quantitative imaging of oil storage in developing crop seeds. Plant Biotechnol J 6:31–45
Nguyen HN, Sabelli PA, Larkins BA (2007) Endoreduplication and programmed cell death in the cereal endosperm. In: Olsen O-A (ed) Endosperm: developmental and molecular biology. Plant cell monographs. Springer, Berlin, pp 21–43
Norstog K (1974) Nucellus during early embryogeny in barley: fine structure. Bot Gaz 135:97–103
Offler CE, McCurdy DW, Patrick JW, Talbot MJ (2002) Transfer cells: cells specialized for a special purpose. Annu Rev Plant Biol 54:431–454

Olsen OA (2004) Nuclear endosperm development in cereals and *Arabidopsis thaliana*. Plant Cell 16:S214–S227

Opsahl-Ferstad HG, Le Deunff E, Dumas C, Rogowsky PM (1997) ZmEsr, a novel endosperm-specific gene expressed in a restricted region around the maize embryo. Plant J 12:235–246

Price PB, Parsons J (1979) Distribution of lipids in embryonic axis, endosperm and hull fractions of barley. J Agric Food Chem 27:813–817

Quesnelle PE, Emery RJN (2007) cis-Cytokinins that pre-dominate in Pisum sativum during early embryogenesis will accelerate embryo growth in vitro. Can J Bot Rev Can Bot 85:91–103

Radchuk V, Borisjuk L, Radchuk R, Steinbiss H-H, Rolletschek H, Broeders S, Wobus U (2006) *Jekyll* encodes a novel protein involved in the sexual reproduction of barley. Plant Cell 18: 1652–1666

Radchuk VV, Borisjuk L, Sreenivasulu N, Merx K, Mock H-P, Rolletschek H, Wobus U, Weschke W (2009) Spatiotemporal profiling of starch biosynthesis and degradation in the developing barley grain. Plant Physiol 150:190–204

Radchuk R, Emery RJN, Weier D, Vigeolas H, Geigenberger P, Lunn JE, Feil R, Weschke W, Weber H (2010a) Sucrose non-fermenting kinase 1 (SnRK1) coordinates metabolic and hormonal signals during pea cotyledon growth and differentiation. Plant J 61:324–338

Radchuk R, Conrad U, Saalbach I, Giersberg M, Emery RJN, Küster H, Nunes-Nesi A, Fernie AR, Weschke W, Weber H (2010b) Abscisic acid deficiency of developing pea embryos achieved by immunomodulation attenuates developmental phase transition and storage metabolism. Plant J 64:715–730

Radchuk V, Weier D, Radchuk R, Weschke W, Weber H (2011) Development of maternal seed tissue in barley is mediated by regulated cell expansion and cell disintegration and coordinated with endosperm growth. J Exp Bot 62:1217–1227

Raghavan V (1986) Embryogenesis in angiosperms. A developmental and experimental study. Cambridge University Press, Cambridge

Reynolds M, Bonnett D, Chapman SC, Furbank RT, Manès Y, Mather DE, Parry MAJ (2011) Raising yield potential of wheat. I. Overview of a consortium approach and breeding strategies. J Exp Bot 62:439–452

Rijavec T, Kovaĉ M, Kladnik A, Chourey PS, Dermastia M (2009) A comparative study on the role of cytokinins in caryopsis development in the maize *miniature1* seed mutant and its wild type. J Integr Plant Biol 51:840–849

Rober-Kleber N, Albrechtová JT, Fleig S, Huck N, Michalke W, Wagner E, Speth V, Neuhaus G, Fischer-Iglesias C (2003) Plasma membrane H+-ATPase is involved in auxin-mediated cell elongation during wheat embryo development. Plant Physiol 131:1302–1312

Röder MS, Kaiser C, Weschke W (2006) Molecular mapping of the shrunken endosperm genes *seg8* and *sex1* in barley (*Hordeum vulgare* L.). Genome 49:1209–1214

Roitsch T, González MC (2004) Function and regulation of plant invertases: sweet sensations. Trends Plant Sci 9:606–613

Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot 54:513–524

Rolletschek H, Weschke W, Weber H, Wobus U, Borisjuk L (2004) Energy state and its control on seed development: starch accumulation is associated with high ATP and steep oxygen gradients within barley grains. J Exp Bot 55:1351–1359

Sabelli PA (2012) Replicate and die for your own good: endoreduplication and cell death in the cereal endosperm. J Cer Sci 56:9–20

Sabelli PA, Larkins BA (2009a) The development of endosperm in grasses. Plant Physiol 149: 14–26

Sabelli PA, Larkins BA (2009b) The contribution of cell cycle regulation to endosperm development. Sex Plant Rep 22:207–219

Salmenkallio M, Sopanen T (1989) Amino acid and peptide uptake in the scutella grains of barley, wheat, rice and maize. Plant Physiol 89:1285–1291

Schaller GE, Kieber JJ, Shiu S-H (2008) Two-component signalling elements and histidyl-aspartyl phosphorelays. The Arabidopsis book. doi:10.1199/tab.0112
Schoonheim PJ, Veiga H, da Costa PD, Friso G, van Wijk KJ, de Boer AH (2007) A comprehensive analysis of the 14-3-3 interactome in barley leaves using a complementary proteomics and two-hybrid approach. Plant Physiol 143:670–683
Schoonheim PJ, da Costa PD, de Boer AH (2009) Dual role for 14-3-3 proteins and ABF transcription factors in gibberellic acid and abscisic acid signalling in barley (*Hordeum vulgare*) aleurone cells. Plant Cell Environ 32:439–447
Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ (2006) The *AUXIN RESPONSE FACTOR 2* gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. Development 133:251–261
Scofield GN, Hirose T, Gaudron JA, Upadhyaya NM, Ohsugi R, Furbank RT (2002) Antisense suppression of the rice sucrose transporter gene, OsSUT1, leads to impaired grain filling and germination but does not affect photosynthesis. Funct Plant Biol 29:815–826
Seiler C, Harshavardhan VT, Rajesh K, Reddy PS, Strickert M, Rolletschek H, Scholz U, Wobus U, Sreenivasulu N (2011) ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. J Exp Bot 62:2615–2632
Shomura A, Izawa T, Ebana K, Ebitani T, Kanegae H, Konishi S, Yano M (2008) Deletion in a gene associated with grain size increased yields during rice domestication. Nat Genet 40: 1023–1028
Smart MG, O'Brien TP (1983) Development of the wheat (Triticum aestivum cultivar Heron) embryo in relation to neighboring tissues. Protoplasma 114(1–2):1–13
Smeekens S (2000) Sugar-induced signal transduction in plants. Annu Rev Plant Physiol Plant Mol Biol 51:49–81
Song X-J, Huang W, Shi M, Zhu M-Z, Lin H-X (2007) A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nat Genet 39:623–630
Sopanen T (1979) Development of peptide transport activity in barley scutellum during germination. Plant Physiol 64:570–574
Sreenivasulu N, Altschmied L, Radchuk V, Gubatz S, Wobus U, Weschke W (2004) Transcript profiles and deduced changes of metabolic pathways in maternal and filial tissues of developing barley grains. Plant J 37:539–553
Sreenivasulu N, Radchuk V, Strickert M, Miersch O, Weschke W, Wobus U (2006) Gene expression patterns reveal tissue-specific signaling networks controlling programmed cell death and ABA- regulated maturation in developing barley seeds. Plant J 47:310–327
Sreenivasulu N, Borisjuk L, Junker BH, Mock H-P, Rolletschek H, Seiffert U, Weschke W, Wobus U (2010a) Barley grain development: toward an integrative view. Int Rev Cell Mol Biol 281:49–89
Sreenivasulu N, Radchuk V, Alawady A, Borisjuk L, Weier D, Staroske N, Fuchs J, Miersch O, Strickert M, Usadel B, Wobus U, Grimm B, Weber H, Weschke W (2010b) De-regulation of abscisic acid contents causes abnormal endosperm development in the barley mutant *seg8*. Plant J 64:589–603
Sugimoto-Shirasu K, Roberts K (2003) "Big it up": endoreduplication and cell-size control in plants. Curr Opin Plant Biol 6:544–553
Swain SM, Ross JJ, Reid JB, Kamiya Y (1995) Gibberellins and pea seed development. Expression of the lh^{i}, *ls* and le^{5839} mutations. Planta 195:426–433
Talbot MJ, Wasteneys G, McCurdy DW, Offler CE (2007) Deposition patterns of cellulose microfibrils in flange wall ingrowths of transfer cells indicate clear parallels with those of secondary wall thickenings. Funct Plant Biol 34:307–313
Tanaka H, Onouchi H, Kondo M, Hara-Nishimura I, Nishimura M, Machida C, Machida Y (2001) A subtilisin-like serine protease is required for epidermal surface formation in Arabidopsis embryos and juvenile plants. Development 128:4681–4689
Taylor IB, Burbidge A, Thompson AJ (2000) Control of abscisic acid synthesis. J Exp Bot 51: 1563–1574

Thiel J, Weier D, Sreenivasulu N, Strickert M, Weichert N, Melzer M, Czauderna T, Wobus U, Weber H, Weschke W (2008) Different hormonal regulation of cellular differentiation and function in nucellar projection and endosperm transfer cells: a microdissection-based transcriptome study of young barley grains. Plant Physiol 148:1436–1452

Thiel J, Müller M, Weschke W, Weber H (2009) Amino acid metabolism at the maternal-filial boundary of young barley seeds: a microdissection-based study. Planta 230:205–213

Thiel J, Hollmann J, Rutten T, Weber H, Scholz U, Weschke W (2012a) Tissue-specific 454 transcriptome sequencing reveals a role for two-component signalling in cellularization and early differentiation of barley endosperm cells. PLoS One 7(7):e41867

Thiel J, Riewe D, Rutten T, Melzer M, Friedel S, Bollenbeck F, Weschke W, Weber H (2012b) Differentiation of endosperm transfer cells of barley – a comprehensive analysis at the micro scale. Plant J 71:639–655. doi:10.111/j.1365-313X.2012.05018.x

Thompson RD, Hueros G, Becker HA, Maitz M (2001) Development and functions of seed transfer cells. Plant Sci 160:775–783

Waterworth WM, West CE, Bray CM (2000) The barley scutellar peptide transporter: biochemical characterization and localization to the plasma membrane. J Exp Bot 51:201–1209

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U (1995) Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell type-specific expression. Plant Cell 7:1835–1846

Weber H, Borisjuk L, Wobus U (1996) Controlling seed development and seed size in *Vicia faba*: a role for seed coat-associated invertases and carbohydrate state. Plant J 10:823–834

Weber H, Borisjuk L, Wobus U (1997) Sugar import and metabolism during seed development. Trends Plant Sci 2:169–174

Weber H, Borisjuk L, Wobus U (2005) Molecular physiology of legume seed development. Annu Rev Plant Biol 56:253–279

Weichert N, Saalbach I, Weichert H, Kohl S, Erban A, Kopka J, Hause B, Varshney A, Sreenivasulu N, Strickert M, Kumlehn J, Weschke W, Weber H (2010) Increasing sucrose uptake capacity of wheat grains stimulates storage protein synthesis. Plant Physiol 152: 698–710

Weigelt K, Kuester H, Rutten T, Fait A, Fernie AR, Miersch O, Wasternack C, Emery RJN, Desel C, Hosein F, Mueller M, Saalbach I, Weber H (2009) ADP-glucose pyrophosphorylase-deficient pea embryos reveal specific transcriptional and metabolic changes of carbon-nitrogen metabolism and stress responses. Plant Physiol 149:395–411

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U (2000) Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. Plant J 21:455–467

Weschke W, Panitz R, Gubatz S, Wang Q, Radchuk R, Weber H, Wobus U (2003) The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. Plant J 33:395–411

Wobus U, Sreenivasulu N, Borisjuk L, Rolletschek H, Panitz R, Gubatz S, Weschke W (2004) Molecular physiology and genomics of developing barley grains. In: Pandalai SG (ed) Recent research developments in plant molecular biology, vol 2. Research Signpost, Kerala, pp 1–29

Yang J, Zhang J, Huang Z, Wang Z, Zhu Q, Liu L (2002) Correlation of cytokinin levels in the endosperms and roots with cell number and cell division activity during endosperm development in rice. Ann Bot 90:369–377

Yang S, Johnston N, Talideh E, Mitchell S, Jeffree C, Goodrich J, Ingram G (2008) The endosperm-specific ZHOUPI gene of Arabidopsis thaliana regulates endosperm breakdown and embryonic epidermal development. Development 135:3501–3509

Zemach A, Kim MY, Silva P, Rodrigues JA, Dotson B, Brooks MD, Zilberman D (2010) Local DNA hypomethylation activates genes in rice endosperm. Proc Natl Acad Sci U S A 107:18729–18734

Zhang L, Cohn NS, Mitchell JP (1996) Induction of a pea cell-wall invertase gene by wounding and its localized expression in phloem. Plant Physiol 112:1111–1117

Zheng YK, Wang Z (2011) Contrast observation and investigation of wheat endosperm transfer cells and nucellar projection transfer cells. Plant Cell Rep 30:1281–1288

Zhou Y, Andriunas F, Offler CE, McCurdy DW, Patrick JW (2010) An epidermal-specific ethylene signal cascade regulates trans-differentiation of transfer cells in *Vicia faba* cotyledons. New Phytol 185:931–943

Zou X, Neuman D, Shen QJ (2008) Interactions of two transcriptional repressors and two transcriptional activators in modulating gibberellin signaling in aleurone cells. Plant Physiol 148:176–186

Chapter 9
Drought Stress Tolerance Mechanisms in Barley and Its Relevance to Cereals

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9.1 Introduction

Crop plants have been systematically improved for higher yields under irrigated conditions in the last decade. However achieving yield stability under semiarid and drought-prone areas remained to be the key target trait to achieve food security. As most of the elite lines among crop plants do not display high resistance to water deficits, there is a need to improve the genetic potential to develop climate-resilient lines. In many parts of the world, drought-prone areas and desertification are growing due to limited and altered pattern of rainfall. This has a tremendous impact on global agricultural production. Among various abiotic stresses, drought is the single most important factor limiting crop production worldwide in the arid and semiarid regions (Boyer and Westgate 2004). Mediterranean regions represent a

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significant area of barley production, also characterized by a long, hot, and dry summer.

Barley is an excellent model plant to study the genetics of drought adaptation, as it is not only an economically important crop but is also known for its high degree of genetic variability for stress tolerance (Sreenivasulu et al. 2010a). Evaluation of barley genotypes for yield stability under drought stress conditions revealed higher genetic plasticity (Khokar et al. 2012; Kalladan et al. 2013). Understanding the molecular mechanisms of plant abiotic stress response is one of the essential areas in plant sciences to derive strategies to develop stress-tolerant cultivars. Plant responses to drought stress are complex and evolved different strategies to alleviate the adverse effects of harsh environments by altering their physiological, molecular, and cellular functions (Witcombe et al. 2008; Blum 2009; Verslues and Juenger 2011). The characterization of many stress-related genes has been preceded by the isolation of candidate genes through traditional way of cloning genes and to extend the approach to large-scale gene expression studies to study drought stress response (Talame et al. 2007). Major progress in this field has come from the application of different "omics" (transcriptome, metabolite, and proteomics) approaches. Application of high-throughput genomics platforms has gained substantial momentum to unravel stress responses. These strategies enabled to identify key stress regulators by deriving regulatory networks (Friedel et al. 2012). Sequence information of complete genomes of model plants and several crop species has significantly enhanced the ability to identify genes associated with drought tolerance. The fundamental molecular mechanisms that underlie the plant abiotic responses are quite conserved among plant species, and therefore, the knowledge gained in the model plants can be extrapolated to improve stress tolerance in crop species.

In the recent past, emphasis has been laid to molecular dissection of drought tolerance by unraveling the molecular physiological mechanisms at the whole-plant level, implementing various phenotyping technologies, attempting towards quantitative trait loci (QTL) cloning through linkage and association mapping or gene discovery by analyzing contrasting lines using transcriptomics and functional genomics strategies (Fleury et al. 2010). The importance of these multifaceted strategies has been reviewed in parts among several crops of cereals such as maize, wheat, rice, and pearl millet (Barnabas et al. 2008; Fleury et al. 2010; Sinclair 2011; Mir et al. 2012; Yadav et al. 2011). However, so far we have not clearly understood the holistic mechanisms for improved seed yield per se under terminal drought, which will be the major topic of the current review. Drought stress responses have been well studied in vegetative tissues under short-term stress response, but little is known about the situation under long-term stress and its relevance under terminal drought (Govind et al. 2011; Seiler et al. 2011). Due to lack of sufficient knowledge from barley, we have also consulted the knowledge revealed in other cereal species to decipher and summarize the drought tolerance mechanisms.

9.2 Plant Responses to Water Deficits

Plants adapt several measures against drought stress. The development of thick cuticle and sunken stomata, development of waxy coating on the leaves and stems, reduction in shoot length, increase in root length that helps in extracting soil moisture, and modification of leaves to scale formation of seeds with low water content are some of the important measures that plants adopt under stress. Drought tolerance strategies can be subdivided into escape and avoidance (Chaves et al. 2003). While escape mechanisms are attributed to early maturity by promoting early flowering, characters associated with avoidance include enhanced root system, stomatal and cuticular resistance, and leaf rolling and unrolling. During avoidance mechanisms, plants maintain high water status by minimizing water loss due to stomatal closure, reduced leaf area, and senescence of older leaves. Drought tolerance represents the ability of the plants to survive and metabolically function to reach the reproductive stage.

Tolerance may involve osmotic adjustments, efficient machinery to quench and scavenge the reactive oxygen species (ROS) formed during stress exposure, and appropriate source–sink readjustments (Sairam and Saxena 2000; Sreenivasulu et al. 2007). Though plants have efficient protective mechanisms against drought stress, bleaching of pigments and a loss of quantum yield occur in many C_3, C_4, and CAM plants. Under drought stress, the photosystem I and II complex, Calvin cycle, and other primary metabolism genes are known to be downregulated (Fig. 9.1). However, the response depends on the genetic plasticity (variable among species and genotypes), the stage of plant development, severity, and duration of stress (Zhu et al. 2005; Harb et al. 2010). Water deficit in the plants also activates accumulation of chemical antioxidants (ascorbic acid and glutathione) and genes

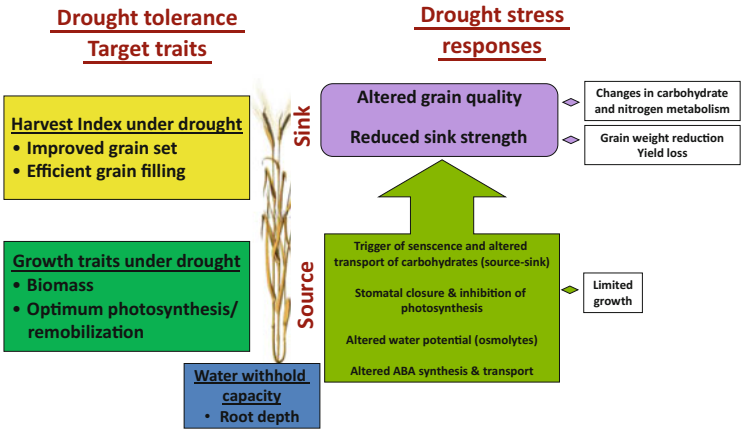

Fig. 9.1 Unraveling molecular–physiological consequences of drought stress responses in cereals to design climate-resilient yield enhancer lines through target trait improvement

associated with antioxidative enzyme machinery (superoxide dismutase, catalase, glutathione S-transferase), synthesis of osmolytes (proline, glycine betaine, trehalose, mannitol, myoinositol), and ion channels and carriers (Sreenivasulu et al. 2007). "Responsive to dehydration" genes encode hydrophilic polypeptides that play a role in protecting cells against drought or salt stress (Shinozaki and Yamaguchi-Shinozaki 1999). Likewise, several fatty acid metabolism-related genes also repair the stress-induced damage in cell membranes. Thus, such genes appear to be important to maintain the membrane fluidity under drought and temperature stresses.

Almost all plants respond to water deficits by producing the phytohormone abscisic acid (ABA) especially in the root system (Bray 2002). Such an accumulation in the roots may help the plants in enhanced water uptake, due to increased hydraulic conductivity. ABA transport to the leaves helps in closure of stomata for reducing the evapotranspiration. This hormone is also involved in the remobilization of stored reserves from vegetative tissues to the grain, during seed filling under drought stress conditions (Yang et al. 2001; Blum 2005). Liu et al. (2005) also showed that under drought stress, ABA has multiple influences on plant development during the reproductive stage.

The sensing of water loss and its response by different plant organs to mediate drought tolerance are regulated through phytohormonal cross talk (Kohli et al. 2013). Among the prominent alteration in hormonal imbalances, ABA accumulates to very high levels under drought stress exposure and mediates mainly the osmotic and ionic adjustments at the cellular level and subsequently reprograms the transcriptome of a cell under stress exposure (Sreenivasulu et al. 2012). Drought stress response is mediated by both ABA-induced and ABA-independent signal transduction (Shinozaki and Yamaguchi-Shinozaki 1999; Sreenivasulu et al. 2007). With the discovery of ABA receptors (components of PYR/PYL receptors) and the downstream component of a type 2C protein phosphatase (PP2C)–SnRK2 protein kinase complex, the ABA signal transduction mechanisms operated through changes in phosphorylation and dephosphorylation events of channel proteins are noted. These events, in conjunction with ABA-induced alteration of cytoplasmic Ca^{2+} and H^{+} concentrations, lead to the closure of K^{+}(inward) and opening of the K^{+}(outward) channels. These changes act as signal for stomatal closure during water deficit and also inhibit H^{+}-ATPase activity, resulting in the reduction of membrane potential. Klimecka and Muszynska (2007) pointed out that Ca^{2+}-dependent protein kinases play a pivotal role in drought stress-related signaling pathways. ABA-regulated gene expression during water deficits is mainly mediated by ABI5 and ABA-responsive-element binding factors, i.e., AREB/ABFs (belongs to bZIP transcription factor family).

9.3 Understanding Drought Stress Response Using Functional Genomics

For a given barley genome of 5.1 Gb, genome-wide physical (95 % covered) and genetic maps have been developed and predicted 26K genes which show significant homology to other plant genomes (The International Barley Genome Consortium). Recent insights of barley genome with emphasis of the posttranscriptional processing (alternative splicing, premature termination codons, abundance of repetitive DNA), high-throughput studies on transcriptome, proteome, and metabolome, and the availability of genome-wide knockout collections and efficient transformation techniques gave new insights into the structural and functional genomics to understand stress tolerance.

Drought tolerance is a complex trait which involves many molecular, biochemical, physiological, phenological, and whole-plant responses that enable plants to withstand stress. From agricultural point of view, drought tolerance essentially means grain yield (Fleury et al. 2010). Because of its complex nature, drought tolerance has to be dissected at different levels to understand the genetic basis of tolerance mechanisms so that we can develop superior genotypes to cope up with the increasing scarcity of water. An upcoming field in plant biology is a systems biology approach which integrates data from different omics such as transcriptomics, metabolomics, and proteomics to identify the molecular targets for crop improvement (Kitano 2002; Salekdeh et al. 2002; Sreenivasulu and Wobus 2013). Such an integrated approach enables to study different processes at the cellular level to unravel cross talk between different signaling components in mediating stress responses at different depth of tissue/organ/organism (Cramer et al. 2011; Jogaiah et al. 2012). Development of drought-tolerant varieties in crops remains far behind compared to other traits due to the diverging drought responses of crop plants across different stages of plant life cycle (Yang et al. 2010). Currently, plant stress responses are studied using either one or a combination of two approaches, mainly transcript and metabolite analysis.

A comparative analysis of barley leaf proteome as affected by drought stress has been carried out recently by Ashoub et al. (2013). They reported the adaptive response of Egyptian barley land races to drought stress using differential gel electrophoresis (DIGE). They observed alterations in proteins related to the energy balance, transcription, protein synthesis, proteins involved in metabolism, and chaperones between the drought-tolerant and susceptible lines. Although transcriptome analysis is widely used to study various abiotic stress responses in model plants, a poor correlation of transcripts with protein profiles or enzyme activities urged the need to combine the transcriptomics with other approaches such as metabolomics or proteomics. Many studies demonstrated that drought tolerance mechanisms differ substantially between genotypes. Thus, exploring intraspecies variation for various tolerant mechanisms through combinatory approaches such as physiology, biochemical responses, genomics, and genetics will identify the important source of material for breeding. In barley, most of

these studies addressed only either source or sink using physiological or biochemical or molecular and in some cases a combination of aforementioned techniques. Talame et al. (2007) monitored the expression changes in leaves of barley subjected to slow drying conditions. They noticed that ~10 % of all transcripts profiled were either up- or downregulated in short-term shock or long-term drought stress conditions. Some of the examples related to identification of key genes using transcriptome analysis have been used to create transgenic plants and validated under field conditions, which include a stress-responsive *NAC* transcription factor (*SNAC*1) (Hu et al. 2006) and late embryogenesis abundant (*LEA*) genes in rice (Xiao et al. 2007). In barley, a transcriptomic approach has been employed to study spike responses to light and drought stresses (Abebe et al. 2010; Mangelsen et al. 2010), as well as near isogenic lines (NILs) differing in nitrogen mobilization during senescence (Jukanti et al. 2008). Homologous genes of different classes participating in LEA biosynthesis, antioxidative pathways, and osmolyte synthesis were identified in transcriptomics experiments comparing wheat lines grown under water-stressed conditions (Aprile et al. 2009; Ergen and Budak 2009; Ergen et al. 2009). Few experiments have reported the use of microarrays for gene expression analysis under water deficit conditions or short duration of drought treatments in barley (Walia et al. 2006; Talame et al. 2007). Unfortunately, some of these studies merely focus on a single genotype during drought stress. Hence, separation of genes associated with drought tolerance from that of drought-responsive genes is not easy. Until now, most of the transcriptomic response has been studied in vegetative tissues, and recently few attempts were made to reveal the transcriptome alterations in developing grains to understand the yield stability including barley under drought (Worch et al. 2011). However, its full implications in understanding source–sink relationships are yet to emerge.

9.4 The Effect of Drought Stress on Flower Initiation and Pollen and Ovary Development

Barley grown in semiarid and Mediterranean region is often coincided with terminal drought conditions that affect the yield (Ceccarelli et al. 2007). Passioura (1996) recognized that promoting flower development under stress is the most important trait conferring drought tolerance to achieve yield stability. In barley, late-flowering phenotypes have been noticed in temperate environments. Early flowering has been identified as an adaptation to short growing seasons and as an escape from the drought stress. Several crops exhibit sensitivity to drought during floral initiation and the premeiotic differentiation of floral parts (Winkel et al. 1997). Using microarray technique, Guo et al. (2009) monitored the changes in gene expression at the transcriptional level in barley leaves during the reproductive stage. They used the drought-tolerant genotype Martin as well as the sensitive genotype Moroc9-75. They observed that 17 genes were expressed constitutively in drought-tolerant

Martin compared with susceptible Moroc9-75 under water deficit and control conditions. Further, they observed that seven annotated genes belong to signaling [calcium-dependent protein kinase (CDPK), membrane steroid binding protein (MSBP)], anti-senescence, and detoxification pathways.

Drought may delay flowering induction or cause total inhibition as pointed out by Winkel et al. (1997). In almost all cereals, meiosis (meiotic stage of plants) is highly sensitive to drought and high temperature (Boyer and Westgate 2004). Due to this, final productivity or yield is reduced up to 75 % in wheat (Saini and Aspinall 1981; Saini and Lalonde 1998) and rice (Sheoran and Saini 1996). Severe water deficit causes pollen sterility in several crops like wheat (Saini et al. 1984; Koonjul et al. 2005) due to abnormalities in microsporogenesis. Sterility of pollen is due to reduced supply of carbohydrates to the anthers and reduced activity of vacuolar and cell wall invertases (Sheoran and Saini 1996; Saini 1997; Koonjul et al. 2005; Oliver et al. 2005). Thus, the signal for pollen sterility in cereals appears to be lowered carbohydrates and reduction in invertase activity (McLaughlin and Boyer 2004).

Besides causing sterility of pollen grains, drought also delays female organ development in maize and other cereals (Damptey et al. 1976; Blum 2000). The ovary has been found to accumulate ABA under prolonged stress (Boyer and Westgate 2004), but it declines once the plants start flowering. Yang et al. (2001) also point out that ABA accumulation in the reproductive structures under stress conditions may inhibit cell division, abort female flower structures, and thus affect grain development. Since water stress causes serious losses of photosynthates, there would be reduced influx of nutrients including carbohydrates to the female reproductive organs (Fig. 9.1). This would ultimately reduce the final productivity in cereals (Makela et al. 2005). The research results of Zinselmeier et al. (1995) revealed that supply of sucrose to the ovaries is important and sucrose can rescue the ovaries from abortion under water deficit conditions. Under drought stress, sucrose may serve not only as a source of substrate for plant survival but also acts as a signaling molecule (Boyer and McLaughlin 2007). Several studies revealed that sucrose and hexose transporters as well sucrose partitioning genes were downregulated in female reproductive organs, associated with ovary abortion. This phenomenon upregulates the genes for the ribosome-inactivating protein (*RIP2*) and phospholipase D (*PLD1*) (McLaughlin and Boyer 2004) triggering senescence and abortion of ovaries. Therefore, such genes are the natural targets for preventing ovary abortion in cereals (Boyer and McLaughlin 2007).

9.5 Carbon Partitioning Between Source–Sink Tissues Under Terminal Drought

Grain yield in cereals is a result of coordinated activities between source and sink tissues. Under optimal conditions, grain growth or seed yield is generally sink limited (Jenner et al. 1991). Sink strength plays a primary role in grain filling of cereals. Water deficit conditions during terminal drought not only reduced the photosynthesis but also triggered senescence and shortened grain filling time (Fig. 9.1). Genotypes which possess remobilization capacity mobilize stem reserves to the grain filling cite (Yang and Zhang 2006a). In cereals like barley, wheat, and maize, pre-anthesis stem reserve accumulation affects flower and grain development (Blum 1998, 2000). Cereal crops store excess carbohydrates in the form of soluble sugars or sugar polymers within the vegetative tissues (Davis et al. 2011). They are also capable of storing nonstructural carbohydrates in the parenchyma cells of stems surrounding the vascular bundles located within internodes. Stem carbohydrates may be stored as soluble sugars such as sucrose, fructans (as in barley, wheat), or starch which is an insoluble polymer of glucose (Halford et al. 2011). Such a whole-plant carbon partitioning may be necessary to buffer the source–sink interaction which may ultimately help to gain yield stability by providing an alternative source of assimilates, when photosynthetic capacity is limited during the period of drought stress. Accumulation of sugars in the stems may also help the plants to pull water from the soil into the vegetative parts of the plants through adjustment of turgor (Fu et al. 2011). These authors have shown that pre-anthesis accumulation of nonstructural carbohydrate reserves in the stem enhances the sink strength of inferior spikelets during grain filling in rice. Reynolds et al. (2011) pointed that optimizing carbon partitioning among vegetative organs (stem) is vital to increase kernel weight. We propose that such a readjustment is based on many interconnecting factors such as photosynthetic efficiency, assimilate competition between organs (newly formed tillers, stem reserve accumulation versus sink strength of developing seeds) and environmental influences such as water and nutrient availability, photoperiod, and temperature. The genetic factors controlling partitioning of current assimilates by basipetal and acropetal movement decide the partitioning strategy either to build stem water soluble carbohydrates or to strengthen sink tissues. This eventually decides the fate of seed filling. Newton et al. (2011) also suggested that one way to increase sink strength (developing seed) is through readjustment of nonstructural carbohydrates in stems of barley. But we need to understand in-depth mechanisms about carbohydrate partitioning at the whole-plant level, so that we can implement strategies that help to create better crops.

Nitrogen (N) application at the spikelet differentiation stage improved the pre-anthesis WSC reserves and sink strength in plants. However, under terminal drought, yield losses in cereals are a result of both source and sink limitations. Yield reduction in barley and other crops even with adequate assimilates made available through artificial feeding to developing grain clearly indicates the role of sink

activity in determining yield under terminal drought (Westgate 1994). Besides the lower number of endosperm cells being the limiting factor of sink strength, the rate of storage product accumulation and duration of seed filling are also identified as other important target traits to increase grain weight under drought (Sreenivasulu et al. 2012).

9.6 Emphasis of Starch Metabolism During Grain Filling Under Terminal Drought

Drought stress affects grain filling in many cereals, resulting in reduced grain weight due to impairment in cell division and reduction in starch accumulation (Nicolas et al. 1985). Wallwork et al. (1998) observed changes in endosperm structure and degradation of storage products in the endosperm of barley when exposed to heat stress. With starch being the predominant form of storage product in barley grain, activities of enzymes involved in conversion of sucrose to starch are the major factors determining sink activity and hence crop yield (Duffus 1992). Among various enzymes involved in starch synthesis, sucrose synthase, which catalyzes the conversion of sucrose to fructose and UDP-glucose, is considered to be one of the important marker enzymes of sink strength in several crops including cereals (Jiang et al. 2012). Its activity was found to be a major determinant of seed filling duration in barley and wheat under both optimal and water deficit conditions (MacLeod and Duffus 1988). A relatively low responsiveness of this enzyme to drought compared to control in maize and in a variety of crops during early grain filling and pollination suggests that its activity may not be a limiting factor for starch synthesis during water-limiting conditions (Sheoran and Saini 1996). On the other hand, reduction in the activity of acid invertase, another enzyme involved in the breakdown of sucrose especially during early stages of seed development in barley (Anderson et al. 2002; Sreenivasulu et al. 2004), was pronouncedly inhibited under water-limited conditions and also in wheat as well as in maize (Zinselmeier et al. 1995; Dorion et al. 1996). Therefore, fine-tuning of different sucrose cleavage pathways as per the requirement in a stage-dependent fashion is an important criterion for regulating seed metabolism under drought (Fig. 9.1).

ADP-glucose pyrophosphorylase (AGPase), an important rate-limiting enzyme of starch synthesis catalyzing the production of ADP-glucose, was found to be negatively affected by drought stress in barley (Seiler et al. 2011), wheat, and potato (Geigenberger et al. 1997). Similarly, reduction in activity of this enzyme was also noticed under heat stress in wheat and in vitro cultured maize (Ahmadi and Baker 2001). Drought stress did not significantly affect the activity of granule-bound starch synthase (GBSS) in wheat when it occurred during the initial stages of seed development but was negatively affected in maize kernels (Ober et al. 1991). A reduction in soluble starch synthase (SSS) activity in wheat under heat stress was correlated with reduction in starch accumulation (Keeling

et al. 1993); however, it was little affected by drought in maize (Dorion et al. 1996). A notable exception to all the above results was reported in a controlled soil-drying experiment carried out by Yang et al. (2003, 2004) in rice and wheat during grain filling. These authors found that activities of sucrose synthase, ADP-glucose pyrophosphorylase, soluble starch synthase, and starch branching enzyme were significantly enhanced under moderate drought and was positively correlated with increased rate of seed starch accumulation resulting in better seed weight compared to control but with reduced seed filling duration. Enhanced seed filling under mild drying was attributed to accumulation of ABA which enhanced sink strength and remobilization of stem reserves. Similarly, a role of ABA in seed filling under terminal drought was also shown by Seiler et al. (2011) and Govind et al. (2011). But terminal drought stress had no effect on the germination of barley seeds, but reduced germination was noticed after the accelerated aging test (Samarah and Alqudah 2011). Worch et al. (2011) monitored and mapped the expression patterns of drought stress-regulated genes in barley during plant ontogeny, and the location of these genes was incorporated into barley SNP linkage map. They pointed out that domestication and breeding have eroded their allelic diversity in the current elite cultivars being used.

9.7 Nitrogen Metabolism in Developing Seeds of Barley Under Terminal Drought and Its Implications on Malting

Another important aspect of terminal drought with respect to grain filling in cereals is altered protein metabolism. Among many factors, seed protein content is the most important one determining the end use of barley for malting. Generally, a low protein content which is usually less than 11.5 % is preferred for malting, as high protein content was found to negatively affect both malt extract and beer quality (Weston et al. 1993). Terminal drought and heat stress are known to increase seed protein content of barley, rendering it unsuitable for malting (Savin and Nicolas 1996). A major reason for increased seed protein content observed under water deficit conditions is due to the fact that starch deposition is more sensitive to drought than protein. Hence, increase in protein content observed under drought is not an increase in protein deposition per se but rather due to the reduction in starch deposition (Brooks et al. 1982). Among seed storage proteins, prolamin (hordein) constitutes more than 50 % of the seed nitrogen in barley and is classified into four groups, namely, B, C, D, and γ based on their electrophoretic mobilities. Among hordeins, the major fraction is constituted by B (70–80 %) and C (10–12 %), while D and γ are considered as minor (Shewry et al. 1985). Studies on the effect of hordein fractions on malting quality revealed that fractions B and D are negatively correlated (Simic et al. 2007); however, no such correlation was found by Shewry et al. (1980) and Riggs et al. (1983). The negative correlation observed

between hordeins and malt extract is attributed to a relatively low starch content of the grain compared to protein and also to the fact that starch granules are embedded into a hordein matrix, thus restricting the access for amylolytic enzymes during malting (Molina-Cano et al. 2000). Further, B and D fractions also reduce the yield of malt extract as they have the tendency to form colloidal aggregates, thus reducing malting quality (Smith and Lister 1983). Therefore, seed protein quality is also important in barley, which is seriously affected under water scarcity.

9.8 QTL Studies: Contribution of Wild Barley for Designing Drought Stress-Tolerant Lines

Wild barley lines are relatively tolerant to both biotic and abiotic stresses. Two QTLs, one located on chromosome 2H and another on 5H, were identified which increased the relative yield by 17 % on average. These two yield QTLs are also associated with heading date (late heading), thus revealing us about the increase in yield potential per se but not by drought escape (Nevo and Chen 2010). They also identified QTLs on chromosome 6H and 7H related to drought tolerance at seedling stage from Israeli wild barley. Current elite crop cultivars are characterized by a limited gene pool due to targeted selection in breeding programs, which reduces their capability to cope with stresses. Crosses of cultivated barley and its progenitor *Hordeum vulgare* ssp. *spontaneum* with favorable effects of the wild barley have the potential to provide alleles with increased drought stress tolerance. To explore intraspecific genetic variation for thousand grain weight (TGW) and seed starch content under terminal drought during the seed filling period, we used a panel of ca. 150 accessions, which included wild relatives, elite lines, several parents of mapping populations, and the introgression line population (BC_3) generated from crossings of elite cultivar Brenda with the wild barley *Hordeum vulgare* subsp. *spontaneum* accession 584 (HS584) as donor parent. Drought tolerance screening trials were performed in both greenhouse and field conditions in two independent years. From the subset of panel containing breeding lines and gene bank accession, haplotype variation was uncovered in the genes encoding sucrose synthase (types I and II) and starch synthase from 17 candidates of starch biosynthesis/degradation genes. The lines which showed dramatic reduction of starch content under terminal drought possess haplotypes H3 (Hv32), H4 (Hs3, Hs5, Hv10), and H5 (OWB-DOM, Hv29, Hv30) from sucrose synthase II gene, and lines possessing haplotype H6 correlate positively to optimum starch accumulation under both control and drought treatments (Worch et al. 2011). We have reported a first step towards the identification of favorable wild barley alleles by constructing a genetic map consisting of drought-regulated ESTs as the basis for the creation of a combined linkage map (Worch et al. 2011). A total of 28 major QTLs (LOD score $\geq$3) were detected with hot-spot QTLs for improved yield/TGW and starch under terminal drought on chromosomes 1H ($DTY_{1.1H,}$) and 2H ($DTY_{2.1H}$) from the

BC_3 doubled haploid introgression lines (ILs) of *Hordeum spontaneum* 584 (BC_3 DH Hs584) populations (Kalladan et al. 2013). Better performing ILs with Hs584 introgression in a hot-spot QTL region (responsible for minimized yield loss under terminal drought) and sensitive ILs (with a severe yield penalty under terminal drought) have been selected to study differential responses to drought stress by transcriptome, metabolite, and enzymatic analyses (Sreenivasulu et al. 2010a, b).

9.9 Transgenic Approaches for Generating Drought Stress-Tolerant Lines

Various stress-responsive genes identified in the past have been subjected to generate transgenic lines in barley and other cereals. Morran et al. (2011) generated transgenic barley plants expressing the *TaDREB2* and *TaDREB3* transcription factors, with both constitutive and drought stress-inducible promoters, and they obtained resistance to drought stress. Expression of other *CBF/DREB* genes, together with a large number of stress-responsive late embryogenesis abundant/cold-regulated/dehydrin genes, also displayed tolerance to water stress (Sakuma et al. 2006). Expression of *HvHVA1* in rice confers dehydration tolerance via maintaining cell membrane stability (Babu et al. 2004), while transgenic overexpression of *HvCBF4* in rice resulted in an increase in tolerance to drought, high salinity, and low temperature without stunting growth (Dong et al. 2006). The barley *HvCBF4* gene was overexpressed in transgenic rice which resulted in increased tolerance to low temperature, drought, and high salinity (Oh et al. 2007). In contrast, in a different rice cultivar, the *HvCBF4* transgene caused enhanced survival to drought but not to high salinity or cold stress (Lourenco et al. 2011). The AREBs/ABFs can bind to an ABA-responsive (ABRE) *cis*-acting element and *trans*-activate downstream gene expression. AREB/ABF-overexpressing barley plants show ABA hypersensitivity and enhanced tolerance to freezing, drought, and salt stress (Furihata et al. 2006). Overexpression of the barley *HVA1* gene in rice led to increased stress tolerance under dehydration stress, compared with the control plants (Rohila et al. 2002). Further, the barley *HVA1* gene under control of a stress-inducible promoter *rd29A* could effectively negate growth retardation under non-stress conditions and confer drought stress tolerance in transgenic mulberry (Checker et al. 2012). From wild barley, the transcription factor *HsDREB1A* has been isolated and incorporated into *Paspalum notatum* using the barley *HVA1* promoter (James et al. 2008). Similarly, *DREB*-like genes were isolated from several species of cereals like rice and wheat and overexpressed in heterologous systems. The results revealed that the transgenics were more drought tolerant compared to their corresponding controls (Hoisington and Ortiz 2008; Perera et al. 2008). Another transcription factor identified using transcriptomics under drought is nuclear transcription factor Y subunit B-1 (*NF-YB1*), and the increased drought tolerance contributed by this

transcription factor was validated both in *Arabidopsis* and maize through transgenic approach. The transgenic maize line overexpressing *ZmNF-YB2* contained higher stomatal conductance and photosynthesis resulting in improved yield due to enhanced drought tolerance (Nelson et al. 2007). Transgenic plants overexpressing these genes were found to have increased drought tolerance under greenhouse conditions. These physiological assessments of transgenic plants for short-term drought survival under glasshouse experiments are not sufficiently conclusive. Therefore, the performance of this wide array of transgenic lines has to be subjected for long-term drought stress assessment under field trials to determine yield stability.

Conclusions

It is of paramount importance to understand the basic physiological and molecular mechanisms underlying drought stress tolerance from the untapped germ plasm to develop climate-resilient lines. It is essential to identify physiological or biochemical traits that are robust, which can enhance the selection of barley breeding lines, so as to use them in drought-prone areas. Terminal drought, being a complex game, affects a plethora of genes in source and sink tissues that encode enzymes associated with chlorophyll metabolism, photosynthesis, and carbohydrate metabolizing pathways (glycolysis, TCA cycle), besides protein degradation and nitrogen metabolism including amino acid transport (Fig. 9.1). Drought tolerance of a species is ultimately measured in terms of seed yield and thousand grain weight. Grain filling in barley and other cereals depends on the potential of carbon synthesis/storage, partitioning, and transport from (1) source to the grain from current photosynthesis and (2) efficient remobilization from reserve pools such as vegetative tissues like stems. Occurrence of drought stress during anthesis and onset of caryopsis development is a very critical factor resulting in impaired grain set, reduced grain weight, and yield loss. This is thought to be at least partly due to a decrease in photosynthetic efficiency and changes in remobilization processes. Ovaries of cereals are normally loaded with glucose and starch on the day of pollination under control conditions (McLaughlin and Boyer 2004). The cell wall and soluble invertase enzymes that metabolize sucrose lose their activity when the delivery of photosynthate is curtailed at low water potentials. Under these conditions, previously accumulated starch is consumed through the activation of amylases (Ruan et al. 2010), resulting often in seed abortion. An alternative source of assimilates are stem reserves stored in the form of sugars, starch, or fructans, which constitute a buffer in case the source capacities are good alternates. These reserves are readily utilized for grain filling under assimilate reduction, when drought occurs during the peak of seed filling in wheat, rice (Yang and Zhang 2006b), and barley (our unpublished results). To create a crop plant that can

(continued)

withstand terminal drought and reduce yield gap, we need to integrate breeding programs, genomics, and systems biology. It is therefore necessary to initiate linking of these complex physiological and biochemical processes to fine-tune source–sink relationships.

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References

Abebe T, Melmaiee K, Berg V, Wise RP (2010) Drought response in the spikes of barley: gene expression in the lemma, palea, awn, and seed. Funct Integr Genomics 10:191–205

Ahmadi A, Baker DA (2001) The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat. Plant Growth Regul 35:81–91

Anderson MN, Asch F, Wu Y, Jensen CR, Naested H, Mogensen VO, Koch KE (2002) Soluble invertase expression is an early target of drought stress during the critical, abortion-sensitive phase of young ovary development in maize. Plant Physiol 130:591–604

Aprile A, Mastrangelo A, De Leonardis A, Galiba G, Roncaglia E, Ferrari F, De Bellis L, Turchi L, Giuliano G, Cattivelli L (2009) Transcriptional profiling in response to terminal drought stress reveals differential responses along the wheat genome. BMC Genomics 10:279

Ashoub A, Beckhaus T, Berberich T, Karas M, Bruggemann W (2013) Comparative analysis of barley leaf proteome as affected by drought stress. Planta 237:771–781

Babu RC, Zhang JX, Blum A, Ho THD, Wu R, Nguyen HT (2004) HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. Plant Sci 166:855–862

Barnabas B, Jager K, Feher A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31:11–38

Blum A (1998) Improving wheat grain filling under stress by stem reserve mobilisation (Reprinted from wheat: prospects for global improvement, 1998). Euphytica 100:77–83

Blum A (2000) Towards standard assays of drought resistance in crop plants. In: Ribaut JM, Poland D (eds) Molecular approaches for the genetic improvement of cereals for stable production in water-limited environments. Proceedings of strategic planning workshop, El Batan, Mexico. pp 29–35

Blum A (2005) Drought resistance, water-use efficiency, and yield potential-are they compatible, dissonant, or mutually exclusive? Aust J Agric Res 56:1159–1168

Blum A (2009) Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. Field Crops Res 112:119–123

Boyer JS, McLaughlin JE (2007) Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion. J Exp Bot 58:267–277

Boyer JS, Westgate ME (2004) Grain yields with limited water. J Exp Bot 55:2385–2394

Bray EA (2002) Abscisic acid regulation of gene expression during water-deficit stress in the era of the *Arabidopsis* genome. Plant Cell Environ 25:153–161

Brooks A, Jenner CF, Aspinall D (1982) Effects of water deficit on endosperm starch granules and on grain physiology of wheat and barley. Funct Plant Biol 9:423–436

Ceccarelli S, Grando S, Baum M (2007) Participatory plant breeding in water-limited environments. Exp Agric 43:1–25

Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought: from genes to the whole plant. Funct Plant Biol 30:239–264

Checker VG, Chhibbar AK, Khurana P (2012) Stress-inducible expression of barley *Hva1* gene in transgenic mulberry displays enhanced tolerance against drought, salinity and cold stress. Transgenic Res 21:939–957

Cramer G, Urano K, Delrot S, Pezzotti M, Shinozaki K (2011) Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biol 11:163

Damptey HB, Coombe BG, Aspinall D (1976) Apical dominance, water deficit and axillary inflorescence growth in *Zea mays*: the role of abscisic acid. Ann Bot 42:1447–1458

Davis SC, Dohleman FG, Long SP (2011) The global potential for Agave as biofuel feedstock. GCB Bioenergy 3:68–78

Dong C, Agarwal M, Zhang Y, Xie Q, Zhu J (2006) Negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc Natl Acad Sci U S A 103:8281–8286

Dorion S, Lalonde S, Saini HS (1996) Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. Plant Physiol 111:137–145

Duffus CM (1992) Control of starch biosynthesis in developing cereal grains. Biochem Soc Trans 20:13–18

Ergen NZ, Budak H (2009) Sequencing over 13 000 expressed sequence tags from six subtractive cDNA libraries of wild and modern wheats following slow drought stress. Plant Cell Environ 32:220–236

Ergen NZ, Thimmapuram J, Bohnert HJ, Budak H (2009) Transcriptome pathways unique to dehydration tolerant relatives of modern wheat. Funct Integr Genomics 9:377–396

Fleury D, Jefferies S, Kuchel H, Langridge P (2010) Genetic and genomic tools to improve drought tolerance in wheat. J Exp Bot 61:3211–3222

Friedel S, Usadel B, von Wirén N, Sreenivasulu N (2012) Reverse engineering: a key component of systems biology to unravel global abiotic stress cross-talk. Front Plant Sci 3:294

Fu Q, Huang Z, Wang Z, Yang J, Zhang J (2011) Pre-anthesis non-structural carbohydrate reserve in the stem enhances the sink strength of inferior spikelets during grain filling of rice. Field Crops Res 103:11–24

Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2006) ABA-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proc Natl Acad Sci U S A 103:1988–1993

Geigenberger P, Reimholz R, Geiger M, Merlo L, Canale V, Stitt M (1997) Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. Planta 201:502–518

Govind G, Seiler C, Wobus U, Sreenivasulu N (2011) Importance of ABA homeostasis under terminal drought stress in regulating grain filling events. Plant Signal Behav 6:1228

Guo P, Baum M, Grando S, Ceccarelli S, Bai G, Li R, von Korff M, Varshney RK, Graner A, Valkoun J (2009) Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. J Exp Bot 60:3531–3544

Halford NG, Curtis TY, Muttucumaru N, Postles J, Mottram DS (2011) Sugars in crop plants. Ann Appl Biol 158:1–25

Harb A, Krishnan A, Madan MR, Ambavaram MR, Pereira A (2010) Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. Plant Physiol 154:1254–1271

Hoisington D, Ortiz R (2008) Research and field monitoring on transgenic crops by the Centro Internacional de Mejoramiento de Maizy Trigo (CIMMYT). Euphytica 164:893–902

Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci U S A 103:12987–12992

James VA, Neibaur I, Altpeter F (2008) Stress inducible expression of the DREB1A transcription factor from xeric, Hordeum spontaneum L. in turf and forage grass (*Paspalum notatum* Flugge) enhances abiotic stress tolerance. Transgenic Res 17:93–104

Jenner C, Ugalde T, Aspinall D (1991) The physiology of starch and protein deposition in the endosperm of wheat. Funct Plant Biol 18:211–226

Jiang Y, Guo W, Zhu H, Ruan Y-L, Zhang T (2012) Overexpression of GhSusA1 increases plant biomass and improves cotton fiber yield and quality. Plant Biotechnol J 10:301–312

Jogaiah S, Govind SR, Tran LS (2012) System biology-based approaches towards understanding drought tolerance in food crops. Crit Rev Biotechnol 33:23–39

Jukanti AK, Heidlebaugh NM, Parrott DL, Fischer IA, McInnerney K, Fischer AM (2008) Comparative transcriptome profiling of near-isogenic barley (*Hordeum vulgare*) lines differing in the allelic state of a major grain protein content locus identifies genes with possible roles in leaf senescence and nitrogen reallocation. New Phytol 177:333–349

Kalladan R, Worch S, Rolletschek H, Harshavardhan VT, Kuntze L, Seiler C, Sreenivasulu N, Röder MS (2013) Identification of quantitative trait loci contributing to yield and seed quality parameters under terminal drought in barley advanced backcross lines. Mol Breed 32:71–99

Keeling PL, Bacon PJ, Holt DC (1993) Elevated temperature reduces starch deposition in wheat endosperm by reducing the activity of soluble starch synthase. Planta 191:342–348

Khokar ML, Teixeira da Silva JA, Spiertz H (2012) Evaluation of barley genotypes for yielding ability and drought tolerance under irrigated and water-stressed conditions. Am Eurasian J Agric Environ Sci 12:287–292

Kitano H (2002) Systems biology: a brief overview. Science 295:1662–1664

Klimecka M, Muszynska G (2007) Structure and function of plant calcium-dependent protein kinases. Acta Biochim Pol 54:1–15

Kohli A, Sreenivasulu N, Lakshmanan P, Kumar PP (2013) The phytohormone crosstalk paradigm takes center stage in understanding how plants respond to abiotic stresses. Plant Cell Rep 32:945–957

Koonjul PK, Minhas JS, Nunes C, Sheoran IS, Saini HS (2005) Selective transcriptional down-regulation of anther invertases precedes the failure of pollen development in water-stressed wheat. J Exp Bot 56:179–190

Liu F, Jensen CR, Andersen MN (2005) A review of drought adaptation in crop plants: changes in vegetative and reproductive physiology induced ABA-based chemical signals. Aust J Agric Res 56:1245–1252

Lourenco T, Saibo N, Batista R, Ricardo CP, Oliveira MM (2011) Inducible and constitutive expression of *HvCBF4* in rice leads to differential gene expression and drought tolerance. Biol Plantarum 55:653–663

MacLeod L, Duffus C (1988) Reduced starch content and sucrose synthase activity in developing endosperm of barley plants grown at elevated temperatures. Funct Plant Biol 15:367–375

Makela P, McLaughlin JE, Boyer JS (2005) Imaging and quantifying carbohydrate transport to the developing ovaries of maize. Ann Bot 96:939–949

Mangelsen E, Wanke D, Kilian J, Sundberg E, Harter K, Jansson C (2010) Significance of light, sugar, and amino acid supply for diurnal gene regulation in developing barley caryopses. Plant Physiol 153:14–33

McLaughlin JE, Boyer JS (2004) Sugar-responsive gene expression, invertase activity and senescence in aborting maize ovaries at low water potentials. Ann Bot 94:675–689

Mir RR, Zaman-Allah M, Sreenivasulu N, Trethowan R, Varshney RK (2012) Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. Theor Appl Genet 125:625–645

Molina-Cano JL, Polo JP, Sopena A, Voltas J, Perez-Vendrell AM, Romagosa I (2000) Mechanisms of malt extract development in barleys from different European regions. II. Effect of barley hordein fractions on malt extract yield. J Inst Brewing 106:117–123

Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, Eliby S, Shirley N, Langridge P, Lopato S (2011) Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnol J 9:230–249

Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstrom DC, Bensen RJ, Castiglioni PP, Donnarummo MG, Hinchey BS, Kumimoto RW, Maszle DR, Canales RD, Krolikowski KA, Dotson SB, Gutterson N, Ratcliffe OJ, Heard JE (2007) Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci U S A 104:16450–16455

Nevo E, Chen G (2010) Drought and salt tolerances in wild relatives for wheat and barley improvement. Plant Cell Environ 33:670–685

Newton A, Flavell A, George T, Philip L, Barry M, Luke R, Cesar R, Joanne R, Brian S, Swanston J, William T, Robbie W, Philip W, Ian B (2011) Crops that feed the world 4, barley: a resilient crop? Strengths and weaknesses in the context of food security. Food Secur 3:141–178

Nicolas ME, Gleadow RM, Dalling MJ (1985) Effect of post-anthesis drought on cell-division and starch accumulation in developing wheat grains. Ann Bot 55:433–444

Ober ES, Setter TL, Madison JT, Thompson JF, Shapiro PS (1991) Influence of water deficit on maize endosperm development: enzyme activities and RNA transcripts of starch and zein synthesis, abscisic acid, and cell division. Plant Physiol 97:154–164

Oh S, Kwon C, Choi D, Song SI, Kim J (2007) Expression of barley *HvCBF4* enhances tolerance to abiotic stress in transgenic rice. Plant Biotechnol J 5:646–656

Oliver SN, Van Dongen JT, Alfred SC, Mamun EA, Zhao X, Saini HS, Fernandes SF, Blanchard CL, Sutton BG, Geigenberger P, Dennis ES, Dolferus R (2005) Cold-induced repression of the rice anther-specific cell wall invertase gene OSINV4 is correlated with sucrose accumulation and pollen sterility. Plant Cell Environ 28:1534–1551

Passioura JB (1996) Drought and drought tolerance. Plant Growth Regul 20:79–83

Perera IY, Hung CY, Moore CD, Stevenson-Paulik J, Boss WF (2008) Transgenic Arabidopsis plants expressing the type 1 inositol 5-phosphatase exhibit increased drought tolerance and altered abscisic acid signaling. Plant Cell 20:2876–2893

Reynolds M, Bonnett D, Chapman SC, Furbank RT, Manes Y, Mather DE, Parry MAJ (2011) Raising yield potential of wheat. I. Overview of a consortium approach and breeding strategies. J Exp Bot 62:439–452

Riggs TJ, Sanada M, Morgan AG, Smith DB (1983) Use of acid gel-electrophoresis in the characterization of B-hordein protein in relation to malting quality and mildew resistance of barley. J Sci Food Agric 34:576–586

Rohila JS, Rajinder K, Wua J, Wua R (2002) Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley *Hva1* cDNA. Plant Sci 163:525–532

Ruan YL, Jin Y, Yang YJ, Li GJ, Boyer JS (2010) Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat. Mol Plant 3:942–955

Saini HS (1997) Effect of water stress on male gametophyte development in plants. Sex Plant Reprod 10:67–73

Saini HS, Aspinall D (1981) Effect of water deficit on sporogenesis in wheat (*Triticum aestivum* L.). Ann Bot 48:623–633

Saini HS, Lalonde S (1998) Injuries to reproductive development under water stress, and their consequences for crop productivity. J Crop Prod 1:223–248

Saini HS, Sedgley M, Aspinall D (1984) Developmental anatomy in wheat of male sterility induced by heat stress, water deficit or abscisic acid. Aust J Plant Physiol 11:243–253

Sairam RK, Saxena DC (2000) Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. Biol Plantarum 43:245–251

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2a, involved in drought-responsive gene expression. Plant Cell 18:1292–1309

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J (2002) A proteomic approach to analyzing drought and salt-responsiveness in rice. Field Crops Res 76:199–219

Samarah N, Alqudah A (2011) Effects of late-terminal drought stress on seed germination and vigour of barley (*Hordeum vulgare* L.). Arch Agron Soil Sci 57:27–32

Savin R, Nicolas ME (1996) Effects of short periods of drought and high temperature on grain growth and starch accumulation of two malting barley cultivars. Aust J Plant Physiol 23:201–210

Seiler C, Harshavardhan VT, Rajesh K, Reddy PS, Strickert M, Rolletschek H, Scholz U, Wobus U, Sreenivasulu N (2011) ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. J Exp Bot 62:2615–2632

Sheoran I, Saini H (1996) Drought-induced male sterility in rice: changes in carbohydrate levels and enzyme activities associated with the inhibition of starch accumulation in pollen. Sex Plant Reprod 9:161–169

Shewry PR, March JF, Miflin BJ (1980) N-terminal amino-acid-sequence of C-Hordein. Phytochemistry 19:2113–2115

Shewry PR, Kreis M, Parmar S, Lew EJL, Kasarda DD (1985) Identification of gamma-type hordeins in barley. FEBS Lett 190:61–64

Shinozaki K, Yamaguchi-Shinozaki K (1999) Molecular responses to drought stress. In: Shinozaki K, Yamaguchi-Shinozaki K (eds) Molecular responses to cold, drought, heat and salt stress in higher plants. R.G. Landes Company, Austin, TX, pp 11–28

Simic G, Sudar R, Lalic A, Jurkovic Z, Horvat D, Babic D (2007) Relationship between hordein proteins and malt quality in barley cultivars grown in Croatia. Cereal Res Commun 35:1487–1496

Sinclair TR (2011) Challenges in breeding for yield increase for drought. Trends Plant Sci 16:289–293

Smith DB, Lister PR (1983) Gel-forming proteins in barley grain and their relationships with malting quality. J Cereal Sci 1:229–239

Sreenivasulu N, Wobus U (2013) Seed-development programs: a systems biology-based comparison between dicots and monocots. Annu Rev Plant Biol 64:189–217

Sreenivasulu N, Altschmied L, Radchuk V, Gubatz S, Wobus U, Weschke W (2004) Transcript profiles and deduced changes of metabolic pathways in maternal and filial tissues of developing barley grains. Plant J 37:539–553

Sreenivasulu N, Sopory SK, Kavi Kishor PB (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene 388:1–13

Sreenivasulu N, Röder MS, Wobus U (2010a) Trockenstress – eine Suche nach den Ursachen und nach neuen Wegen zur Züchtung trockentoleranter Getreide. GenomXPress 4:4–6

Sreenivasulu N, Borisjuk L, Junker BH, Mock HP, Rolletschek H, Seiffert U, Weschke W, Wobus U (2010b) Barley grain development: toward an integrative view. Int Rev Cell Mol Biol 281:49–89

Sreenivasulu N, Harshavardhan VT, Govind G, Seiler C, Kohli A (2012) Contrapuntal role of ABA: does it mediate stress tolerance or plant growth retardation under long-term drought stress? Gene 506:265–273

Talame V, Ozturk NZ, Bohnert HJ, Tuberosa R (2007) Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. J Exp Bot 58:229–240

Verslues PE, Juenger TE (2011) Drought, metabolites, and *Arabidopsis* natural variation: a promising combination for understanding adaptation to water-limited environments. Curr Opin Plant Biol 14:240–245

Walia H, Wilson C, Wahid A, Condamine P, Cui X, Close TJ (2006) Expression analysis of barley (*Hordeum vulgare* L.) during salinity stress. Funct Integr Genomics 6:143–156

Wallwork MAB, Jenner CF, Logue SJ, Sedgley M (1998) Effect of high temperature during grain-filling on the structure of developing and malted barley grains. Ann Bot 82:587–599
Westgate ME (1994) Seed formation in maize during drought. In: Westgate ME (ed) Physiology and determination of crop yield. American Society of Agronomy, Madison, WI
Weston DT, Horsley RD, Schwarz PB, Goes RJ (1993) Nitrogen and planting date effects on low-protein spring barley. Agron J 85:1170–1174
Winkel T, Renno JF, Payne WA (1997) Effect of the timing of water deficit on growth, phenology and yield of pearl millet [*Pennisetum glaucum* (L.) R. Br.] grown in Sahelian conditions. J Exp Bot 48:1001–1009
Witcombe JR, Hollington PA, Howarth CJ, Reader S, Steele KA (2008) Breeding for abiotic stress for sustainable agriculture. Philos Trans R Soc B Biol Sci 363:703–716
Worch S, Rajesh K, Harshavardhan VT, Pietsch C, Korzun V, Kuntze L, Börner A, Wobus U, Röder MS, Sreenivasulu N (2011) Haplotyping, linkage mapping and expression analysis of barley genes regulated by terminal drought stress influencing seed quality. BMC Plant Biol 11:1
Xiao B, Huang Y, Tang N, Xiong L (2007) Over-expression of a LEA gene in rice improves drought resistance under the field conditions. Theor Appl Genet 115:35–46
Yadav RS, Sehgal D, Vadez V (2011) Using genetic mapping and genomics approaches in understanding and improving drought tolerance in pearl millet. J Exp Bot 62:397–408
Yang J, Zhang J (2006) Grain filling of cereals under soil drying. New Phytol 169:223–236
Yang J, Zhang J, Wang Z, Zhu Q, Wang W (2001) Hormonal changes in the grains of rice subjected to water stress during grain filling. Plant Physiol 127:315–323
Yang J, Zhang J, Wang Z, Liu L, Zhu Q (2003) Postanthesis water deficits enhance grain filling in two-line hybrid rice. Crop Sci 43:2099–2108
Yang J, Zhang J, Wang Z, Xu G, Zhu Q (2004) Activities of key enzymes in sucrose-to-starch conversion in wheat grains subjected to water deficit during grain filling. Plant Physiol 135:1621–1629
Yang SJ, Vanderbeld B, Wan JX, Huang YF (2010) Narrowing down the targets: towards successful genetic engineering of drought-tolerant crops. Mol Plant 3:469–490
Zhu X, Gong H, Chen G, Wang S, Zhang C (2005) Different solute levels in two spring wheat cultivars induced by progressive field water stress at different developmental stages. J Arid Environ 62:1–14
Zinselmeier C, Westgate ME, Schussler JR, Jones RJ (1995) Low water potential disrupts carbohydrate metabolism in maize (*Zea mays* L.) ovaries. Plant Physiol 107:385–391

Chapter 10
Response to Viral Pathogens

Frank Ordon and Thomas Kühne

10.1 Introduction

Virus diseases have gained evident worldwide importance in barley production during the last decades due to high losses in yield and quality and the fact that no direct countermeasures are available to combat viruses. After infection no curative methods are available and only preventive control is efficient in avoiding viral epidemics in case of insect-transmitted viruses while no chemical measures are available to reduce yield losses caused by soilborne viruses. Breeding for virus resistance is therefore of special importance to ensure sustainable barley production in the expanding area of fields infested with soilborne viruses and to facilitate an environmental and consumer-friendly barley production in case of insect-transmitted viruses.

Although barley (*Hordeum vulgare* L.) is a host for more than 50 different plant viruses (http://books.google.de/books?id=K6jAYYMvElcC&printsec=frontcover&hl=de#v=onepage&q&f=false), so far only a very few cause economically relevant diseases. These are the different viruses and strains of the Barley yellow dwarf complex [*Barley yellow dwarf virus* (BYDV), *Cereal yellow dwarf virus* (CYDV)], a strain of *Wheat dwarf virus* (WDV), and the two related soilborne pathogens *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV).

Classical selection schemes, e.g., the pedigree selection scheme, are still today the backbone of breeding for resistance to the abovementioned viruses in barley.

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However, numerous biotechnological approaches are being increasingly employed in breeding for virus resistance: (1) cell and tissue culture techniques facilitate the transfer of virus resistance from the secondary or tertiary gene pool into cultivated barley, (2) the selection process is considerably enhanced by employing doubled haploid (DH) technologies, (3) molecular markers pave the way to efficient marker-based selection procedures, (4) advances in genome sequencing facilitate a faster isolation of resistance genes, and (5) in the near future allele-based breeding for virus resistance will become a new powerful tool [for overview cf. Palloix and Ordon (2011)]. Knowledge on these viruses, sources and genetics of resistance, and its mode of action as well as the possibilities of molecular breeding for virus resistance in barley is briefly reviewed.

10.2 Important Viral Pathogens on Barley

10.2.1 Barley Yellow Dwarf Virus and *Cereal Yellow Dwarf Virus*

Yellow dwarf disease, a ubiquitous virus disease of cereal crops worldwide, is caused by a group of related single-stranded RNA viruses with a genome of 5.3–5.7 kb in size. Their isometric particles, about 25 nm in diameter, are transmitted obligatorily in a persistent, circulative, non-propagative manner by several species of grass-feeding aphids. Most important vectors are *Rhopalosiphum padi*, *Sitobion avenae*, and *R. maidis*. This group of viruses includes three species in the genus *Luteovirus*, called *Barley yellow dwarf virus* (BYDV)-PAV, BYDV-PAS, and BYDV-MAV, while two species known as *Cereal yellow dwarf virus* (CYDV)-RPS and CYDV-RPV belong to the genus *Polerovirus*. Another three species within this virus complex, BYDV-GPV, BYDV-RMV, and BYDV-SGV, also classified as members of the *Luteoviridae* family, have not yet been assigned to any genus (http://ictvonline.org/virusTaxonomy.asp?version=2009). The experimental host range of these viruses consists of more than 150 species of *Poaceae*. Although they induce almost identical symptoms in common host plants, the viruses are characterized by somewhat higher specificity in vector relations. No isolate is known so far that is able to infect dicot plants. PAV is the best studied BYDV; it is most widespread and usually causes most severe symptoms. Infected plants are stunted; leaves may appear water-soaked and develop chlorotic stripes, blotches, or mottle starting at the tip. Leaf chlorosis is often accompanied in barley by a brilliant yellow color. Consequently, infection with BYDV has significant effects on plant height, number of tillers per plant, and grain yield (Lister and Ranieri 1995). Mixed infections of single plants may occur and can cause a significant increase in disease severity compared to single infections as demonstrated for BYDV-PAV and BYDV-RPV (Baltenberger et al. 1987). Average yield losses of winter barley attributable to natural barley yellow dwarf disease are

generally difficult to estimate but are reported to range between 11 and 33 % (Miller and Rasochova 1997). However, due to reduced winter hardiness of virus-infected plants, losses can be even higher.

10.2.2 *Wheat Dwarf Virus*

Wheat dwarf virus (WDV) is a member of the genus *Mastrevirus* in the family *Geminiviridae*. It was found to infect a wide range of grasses in the family of *Poaceae*, including barley. The virus was first detected in Czechoslovakia (Vacke 1961). Today WDV is widely distributed in Central and Northern Europe, and it has been recently reported as well from China and Turkey (Ramsell et al. 2009). The virus has a monopartite single-stranded circular DNA genome of about 2.7 kb. The nucleic acid is encapsidated within virus particles with a characteristic twinned morphology of about 38 nm in length and 22 nm in diameter. Virus particles are transmitted to plants by the leafhopper *Psammotettix alienus* in a circulative, non-propagative manner (http://www.agls.uidaho.edu/ebi/vdie/descr883.htm).

Although originally identified as a wheat-affecting agent, two strains of WDV preferentially infecting barley and oats, respectively, have been described (Lindsten and Vacke 1991; Schubert et al. 2007). While the genomes of the wheat and the barley strains share an average of 84 % genome-wide nucleotide sequence identity, the oat strain is more distantly related showing approximately 70 % genome identity. Under natural conditions the strains are restricted to their cereal host species, albeit based on the present knowledge (Ramsell et al. 2009) it seems likely that both the wheat and the barley strain of WDV can, at least occasionally, infect the other's preferred host, too. The disease symptoms on barley plants are almost indistinguishable from those of a BYDV infection. Yield losses due to WDV infections are estimated comparable to those induced by barley yellow dwarf disease.

10.2.3 *Barley Yellow Mosaic Virus* and *Barley Mild Mosaic Virus*

Yellow mosaic disease in barley crops was first reported from Japan (Ikata and Kawai 1940), and the causal agent was designated *Barley yellow mosaic virus* (BaYMV). Later the same disease was observed in numerous countries in Europe and East Asia (Kühne 2009), and it turned out that two viruses either separately or in combination may induce the same type of symptoms (Huth and Adams 1990). BaYMV and BaMMV are identical in morphology. They have filamentous particles with two modal lengths of 500–600 nm and 250–300 nm, containing two species of ssRNA (Huth et al. 1984). Determination of the complete nucleotide sequences

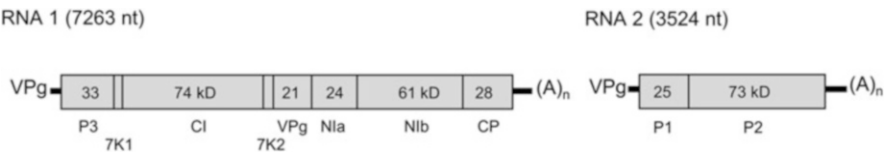

Fig. 10.1 Genome organization of *Barley mild mosaic virus*. Each RNA codes for a single polyprotein, which is autocatalytically cleaved to yield several functional proteins (Kühne 2009)

indicated that RNA1 and RNA2 of BaMMV (Dessens et al. 1995; Kashiwazaki 1996; Meyer and Dessens 1996; Subr et al. 2000; Timpe and Kühne 1994) have similar genome organization, but only a low level of sequence identity to the RNAs of BaYMV (Davidson et al. 1991; Kashiwazaki et al. 1990, 1991; Peerenboom et al. 1992). Thus, the two viruses are related but distinct members of the genus *Bymovirus* in the family *Potyviridae*. The schematic genome organization of bymoviruses, exemplified for BaMMV, is presented in Fig. 10.1.

Under natural conditions BaYMV and BaMMV are transmitted by the root-inhabiting fungal-like plasmodiophorid *Polymyxa graminis* (Adams et al. 1988). This microorganism is distributed worldwide (Anonymous 2011; Thompson et al. 2011) and primarily multiplies in the roots of grass and cereal species. It is an obligate, intercellular parasite with thick-walled resting spores and motile primary and secondary zoospores. The fungal vector provides protection against unfavorable environmental conditions and during long rotations of non-host crops and allows the viruses to persist almost indefinitely once a field has become infested (Adams et al. 1993; Kanyuka et al. 2003). Particles of BaMMV have been detected by electron microscopy inside the zoospores and zoosporangial plasmodia, but this could not be demonstrated for resting spores (Chen et al. 1991). Therefore, the question of whether spores contain bymoviruses as intact virions or intermediate forms (e.g., infectious nucleoproteins) is not conclusively answered yet. On the basis of the ecological requirements and rDNA sequences of distinct *P. graminis* isolates, five special forms have been proposed (Legreve et al. 2002). Two of them, the formae speciales *tepida* and *temperata* infect barley plants (Vaianopoulos et al. 2007a).

BaYMV and BaMMV frequently coexist in infested fields, thus leading to single or mixed infected plants. Significant yield losses up to 50 % are observed only for winter barley crops. Infected plants produce less grain and are more susceptible than healthy plants to winter killing (Huth 1988; Huth and Lesemann 1978; Plumb et al. 1986). In Japan at least seven strains of BaYMV and two strains of BaMMV are known (Kashiwazaki et al. 1989; Sotome et al. 2010), while in Europe in general two strains of BaYMV and two of BaMMV have been described (Habekuss et al. 2008a).

The virus vector *P. graminis* is a ubiquitous microorganism. Since chemical measures to eliminate the vector are neither effective nor acceptable for ecological reasons, the only feasible opportunity to control the barley yellow mosaic disease is breeding resistant cultivars.

10.3 Resistance Against Viral Pathogens

No complete resistance against BYDV/CYDV is known in barley but different genes conferring tolerance have been identified. The first was *ryd1*, detected in the spring barley cultivar "Rojo" (Suneson 1955). This gene was not used in barley breeding due to its low efficiency. Furthermore, *Ryd2* and *Ryd3*, with similar effects against BYDV-PAV and BYDV-MAV, were identified in Ethiopian landraces (Schaller et al. 1964; Niks et al. 2004). The effect of the semidominant *Ryd2* gene located on chromosome 3HL (Collins et al. 1996) depends on the genetic background, the environmental conditions, and the virus isolate (Schaller et al. 1964; Schaller 1984). *Ryd2* has been reported to reduce the virus titer of BYDV-PAV and BYDV-MAV in young plants (Skaria et al. 1985; Baltenberger et al. 1987; Chalhoub et al. 1995; Sip et al. 2006), but no differences in the virus titer of plants carrying *Ryd2* or *ryd2*, respectively, concerning BYDV-PAV were observed in studies with older plants (Skaria et al. 1985; Scheurer et al. 2000). Besides this, different alleles may be present at this locus (Catherall et al. 1970; Chalhoub et al. 1995). *Ryd3* is explaining about 75 % of the phenotypic variance of BYD tolerance in the cross "Vada" × "L94" and has been mapped to chromosome 6H (Niks et al. 2004). Furthermore, in association genetics studies, a large-effect gene for BYD tolerance was detected in European spring barley cultivars on chromosome 2H (Kraakman et al. 2006). In addition to these loci, minor effect QTLs for tolerance against BYDV have been mapped to chromosome 2HL (Scheurer et al. 2001) and other chromosomes (Toojinda et al. 2000). Recently, a gene called $Ryd4^{Hb}$ conferring complete resistance to BYDV-PAV has been transferred from *Hordeum bulbosum* to cultivated barley (Scholz et al. 2009).

With respect to *Wheat dwarf virus*, intensive screening programs for resistance have been conducted (Vacke and Cibulka 2001; Bukyayová et al. 2006), but so far some tolerance to WDV has been only observed in the winter barley cv. "Post" (Habekuss et al. 2008b), which is also the donor for the BYDV-QTL located on chromosome 2HL (Scheurer et al. 2001). Up to now, nothing is known about the genetics of this tolerance.

Concerning resistance to BaMMV/BaYMV, extensive screening programs have been conducted in Japan (e.g., Takahashi et al. 1970) and after the discovery of the disease in Germany in 1978 (Huth and Lesemann 1978) also in Europe (e.g., Ordon et al. 1993). In these studies it turned out that resistance to BaMMV/BaYMV, which in contrast to BYDV is in general a complete resistance — with the exception of *rym7* (Graner et al. 1999a) — is quite frequent within the primary gene pool of barley but that genotypic differences are present with respect to the reaction against the different viruses and virus strains (for overview cf. Ordon et al. 2009). In subsequent genetic analysis, it was shown that resistance is due to different recessive resistance genes (Götz and Friedt 1993; Ordon and Friedt 1993) in *H. vulgare*. In contrast to this, two dominant resistance genes have been detected in *H. bulbosum* [Table 10.1, Ruge et al. (2003), Ruge-Wehling et al. (2006), for overview cf. Ordon et al. (2009)], and recently the first dominant resistance gene

Table 10.1 List of mapped major virus resistance genes in barley (mod. and updated according to Ordon 2009)

Resistance gene	Chromosomal location	Reference(s)
***Barley stripe mosaic virus* (BSMV)**		
Rsm	7HS	Edwards and Steffenson (1996)
***Barley yellow dwarf virus* (BYDV)**		
Ryd2	3HL	Collins et al. (1996), Paltridge et al. (1998), Ford et al. (1998)
Ryd3	6H	Niks et al. (2004)
$Ryd4^{Hb}$	3HL	Scholz et al. (2009)
***Barley yellow mosaic virus* (BaYMV), *Barley mild mosaic virus* (BaMMV)**		
rym1	4HL	Okada et al. (2004)
rym3	5HS	Saeki et al. (1999), Werner et al. (2003a)
rym4	3HL	Graner and Bauer (1993), Ordon et al. (1995), Weyen et al. (1996), Stein et al. (2005), Kanyuka et al. (2005), Werner et al. (2005), Stracke et al. (2007), Tyrka et al. (2008)
rym5	3HL	Graner et al. (1999b), Pellio et al. (2005), Stein et al. (2005), Kanyuka et al. (2005), Stracke et al. (2007), Tyrka et al. (2008)
rym7	1HS	Graner et al. (1999a)
rym8	4HL	Bauer et al. (1997)
rym9	4HL	Bauer et al. (1997), Werner et al. (2000, 2005)
rym10	3HL	Graner et al. (1995)
rym11	4HL	Bauer et al. (1997), Nissan-Azzous et al. (2005), Werner et al. (2005), Luepken et al. (2013)
rym12	4HL	Graner et al. (1996)
rym13	4HL	Werner et al. (2003b), Humbroich et al. (2010)
$Rym14^{Hb}$	6HS	Ruge et al. (2003)
rym15	6H	Le Gouis et al. (2004)
$Rym16^{Hb}$	2HL	Ruge-Wehling et al. (2006)
Rym17	3H	Kai et al. (2012)
rym18	4H	Kai et al. (2012)

Rym17 has been identified also in *H. vulgare* (Kai et al. 2012). In contrast to BYDV and especially WDV, there is a broad genetic variation available in barley concerning resistance to BaMMV and BaYMV.

10.4 Resistance Mechanisms

Up to now, nothing is known about genes and respective mechanisms involved in tolerance against BYDV/CYDV or WDV, but with respect to BaMMV/BaYMV the *Rym4*/*Rym5* locus has been isolated and *rym4* and *rym5* have been identified as allelic forms of the wild-type gene coding for the eukaryotic translation initiation factor 4E (eIF4E) (Kanyuka et al. 2005; Stein et al. 2005). In the last decade a

number of recessive potyvirus resistance genes have been isolated from both monocotyledonous and dicotyledonous plant species identifying eIF4E or its isoform as the host determinant (Diaz-Pendon et al. 2004; Maule et al. 2007). The factor eIF4E is a crucial component in the highly regulated process of protein synthesis in eukaryotic cells. It interacts with the m^7GppN cap group at the 5′ end of mature eukaryotic mRNAs. The eIF4E protein binds with its partner eIF4G to form the eIF4F complex, which serves as a scaffold for the assembly of initiation factors eIF4A, eIF4B, eIF3, and poly(A)-binding protein (Monzingo et al. 2007). This protein-mRNA complex recruits the 40S ribosome with its attendant initiation factors prior to scanning for the initiator AUG codon. Besides its role as a translation initiating factor, eIF4E can also function as a translational repressor (Rhoads 2009).

The first evidence that eIF4E or its isoform eIF(iso)4E may represent a crucial plant factor for multiplication of potyviruses was obtained when interactions between *Turnip mosaic virus*-encoded proteins and proteins of *Arabidopsis thaliana* were investigated with the yeast two-hybrid system (Wittmann et al. 1997). Other in vitro binding assays demonstrated that the potyviral protein, which is covalently linked to the 5′ end of the viral RNA (VPg in Fig. 10.1), interacts with eIF4E or its isoform of the host plant (Leonard et al. 2000). Potyviral VPg proteins apparently have two spatially separated binding sites on eIF4E for optimal interaction. The precise contact points appear to be optimized for each virus/host pair by coevolution, because they occur at different positions on the folded eIF4E molecule (Monzingo et al. 2007). They seem to be distinct from the contact point for the cap structure of plant mRNAs (Michon et al. 2006). Nucleotide substitutions in the coding sequence for eIF4E or VPg leading to conformationally altered proteins may prevent or restore their compatible interaction and in this way inhibit or enable virus multiplication in the plant.

An analysis of 1,090 barley landraces and cultivars from 84 countries recently revealed an exceptionally high nucleotide diversity in the coding sequence of eIF4E, which was to observe neither for an adjacent gene nor for the isoform eIF(iso)4E. A total of 47 haplotypes, including *rym4* and *rym5*, were identified. This unexpectedly high diversity gives hint to a strong positive selection acting on the eIF4E gene suggesting that this factor may play a role in barley adaptation to local habitats. The majority of barley genotypes reflecting the different eIF4E haplotypes are still to be characterized for resistance against BaMMV and BaYMV (Stracke et al. 2007; Hofinger et al. 2011).

Most of the commercial barley cultivars in Europe carry the *rym4* gene conferring resistance to both BaMMV and BaYMV. However, in the late 1980s, a new pathotype designated BaYMV-2, which is able to overcome the *rym4*-controlled resistance, was first detected in Germany and later on also in other European countries. It has been shown that the ability of European BaYMV isolates to infect *rym4*-carrying plants is correlated with a base substitution at nucleotide (nt) position 4,094 of RNA1 (numbering according to EMBL-Bank accession number X69757) leading to an exchange of lysine to asparagine at amino acid (aa) position 132 of the deduced VPg protein (Kühne et al. 2003; Vaianopoulos

et al. 2007b). This pathotype is now widespread in Germany and occurs in nearly all regions with intensive barley production (Wellie-Stephan et al. 2010). Genotypes with the haplotype *rym5* of eIF4E cannot be infected neither by the original strains of BaYMV and BaMMV nor by the new pathotype BaYMV-2. First varieties carrying the *rym5* gene were registered in the late 1990s, and again, shortly after their cultivation resistance was overcome in France and Germany by a new pathotype of BaMMV (Habekuss et al. 2008a; Kanyuka et al. 2004). Sequence analyses of several of these resistance-breaking BaMMV isolates revealed various substitutions of one or a few aa residues in the RNA1-encoded VPg protein. These replacements obviously produce changes in the spatial structure of the molecule in a way that the viral protein fits again to the translation initiation factor in barley cells, thus enabling virus multiplication.

10.5 Molecular Breeding for Virus Resistance in Barley

With respect to the insect-transmitted BYDV/CYDV and WDV, the availability of viruliferous aphids and leafhoppers, respectively, is a prerequisite for efficient breeding for resistance/tolerance on the phenotypic level while effective selection procedures against BaMMV/BaYMV require uniformly infested fields. In addition, it has to be taken into account that rearing of viruliferous insects and artificial infection is difficult to integrate into applied barley breeding schemes. With respect to BaMMV/BaYMV, symptom development is strongly influenced by the climatic conditions during winter and spring time leading to the fact that a reliable selection for virus resistance on the phenotypic level cannot be conducted each year. Therefore, molecular markers are efficient tools in breeding for virus resistance. Respective markers are available for many major genes conferring tolerance or resistance to BYDV or the BaMMV/BaYMV complex, while up to now no molecular markers are available for tolerance to WDV, but for resistance against *Barley stripe mosaic virus* (BSMV) which is of inferior agronomic importance (Table 10.1). In addition, quantitative trait loci (QTL) for BYDV tolerance have been identified (Scheurer et al. 2001; Toojinda et al. 2000; Kraakman et al. 2006) and may be also employed in marker-assisted selection procedures. An overview of barley virus resistance genes, selectable by molecular markers, together with their chromosomal location is given in Table 10.1.

These markers together with doubled haploid techniques facilitate a reliable selection for virus resistance in barley, e.g., doubled haploid populations can be screened by respective markers directly in vitro and only those plantlets carrying the resistance-encoding allele have to be transferred to the greenhouse (for overview on molecular breeding for virus resistance, cf. Friedt and Ordon 2007; Ordon et al. 2009; Palloix and Ordon 2011).

However, virus resistance is often identified in rather unadapted germplasm (e.g., Ordon and Friedt 1994). Therefore, long-lasting backcrossing procedures are needed to combine virus resistance with superior agronomic performance.

This holds especially true in case recessive resistance genes have to be incorporated. Here, a selfing generation is needed after each backcross to identify homozygous recessive genotypes on the phenotypic level. In contrast to this, the recessive resistance-encoding allele can be directly followed by a codominant marker or a dominant one showing an additional fragment linked to the resistance-encoding allele (Ordon et al. 2003, 2009), thus saving one generation per backcrossing cycle. This process can be further enhanced if in parallel the genomic portion of the recurrent parent is determined, e.g., by efficient high-throughput single nucleotide polymorphism (SNP) genotyping (Close et al. 2009; Comadran et al. 2012), as in many species (e.g., Uptmoor et al. 2006) a strong deviation from the theoretically expected portion of 75 % in BC_1 was observed.

Molecular markers furthermore facilitate efficient pyramiding of resistance genes especially in combination with doubled haploids (Werner et al. 2005). With respect to BaMMV/BaYMV, gene pyramiding may become of special importance in the future as most of the resistance genes (Table 10.1) have been overcome already by new pathotypes of BaYMV or BaMMV (e.g., Habekuss et al. 2008a). This approach, which may be conducted following strategies involving one or two DH line production steps (Werner et al. 2005), facilitates the extended use of respective resistance genes in barley breeding. As an example, the combination of *rym5* (effective in Europe against BaMMV, BaYMV, and BaYMV-2) with *rym9* (effective against BaMMV, BaMMV-SIL, and BaMMV-Teik) will result in complete resistance to all strains known in Europe up to now. Besides extending the usability of resistance genes, pyramiding may also result in a higher level of resistance as has been recently shown for BYDV (Riedel et al. 2011). In these studies, *Ryd2*, *Ryd3*, and the QTL located on chromosome 2H derived from cv. "Post" were combined using DH lines and molecular markers, and it turned out that those lines carrying a combination of *Ryd2* and *Ryd3* showed a significantly reduced virus titer, i.e., a combination of these loci has resulted in quantitative resistance to BYDV in contrast to tolerance encoded by these loci singly (Fig. 10.2).

10.6 Future Perspectives

Breeding for virus resistance has achieved considerable success in the past, and already today molecular markers facilitate efficient breeding for virus resistance/tolerance in barley on the molecular level. Recently, the genomic sequence of monocot species, i.e., *Brachypodium* and *Sorghum*, has become available in addition to the rice genome, and efficient tools for exploiting the synteny between these species and barley have been developed, which together with the constantly rising sequence information in barley itself (Mayer et al. 2011) will lead to an enhanced isolation of virus resistance genes in this species. These tools together with the availability of high-density maps (Close et al. 2009; Sato et al. 2009; Comadran et al. 2012) available in barley have been already successfully employed in identifying a candidate gene for *rym11* (Luepken et al. 2013, which has been recently

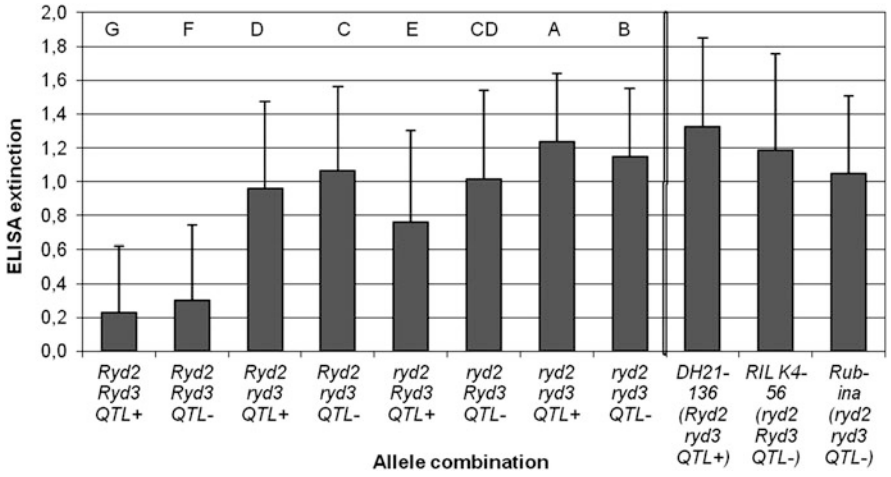

Fig. 10.2 Average ELISA extinction (405 nm) and standard deviation in DH lines of the population "RIL K4-56" × "DH21-136" carrying different allele combinations at the *Ryd2* locus, *Ryd3* locus, and the QTL on chromosome 2H determined after experimental BYDV-PAV inoculation on four locations and in 2 years in field trials. Different letters indicate significant differences (Tukey test, $\alpha = 0.05$). Data of parental lines and the susceptible standard are shown for comparison (Riedel et al. 2011)

isolated (Yang et al. 2014). In the near future, the process of isolating virus resistance genes in barley via map-based cloning will be further enhanced as the first physical, genetical, and functional sequence assembly of the barley genome has been published (International Barley Genome Sequencing Consortium IBSC 2012) and the complete sequence is expected soon. The isolation of genes involved in virus resistance will transfer breeding to the allele level facilitating the identification of novel alleles and their directed use in molecular breeding strategies in order to enhance virus resistance. The use of these alleles mainly derived from exotic germplasm can be fostered by marker-assisted backcrossing for the gene of interest simultaneously with the enhanced elimination of the donor background by genotyping using high-throughput SNP technologies, e.g., the 9k iSelect chip in barley (Comadran et al. 2012). However, respective alleles may also be transferred directly to high-yielding cultivars or be combined using new advances in gene technology like zinc-finger nucleases (Shukla et al. 2009) or the TALEN technology (Zhang et al. 2013). Gene technology does not only facilitate the enhanced use of the allelic diversity present with respect to virus resistance genes within the barley gene pool (allele replacement) but also to create new virus resistance, e.g., using small interfering RNAs (Sun et al. 2008; Prins et al. 2008).

In summary, all these advances in biotechnology will improve breeding for resistance to viruses in barley and will enable plant breeding to react in a directed and fast manner to the challenges arising from new virus diseases and virus strains in this crop.

References

Adams MJ, Swaby AG, Jones P (1988) Confirmation of the transmission of barley yellow mosaic virus (BaYMV) by the fungus Polymyxa graminis. Ann Appl Biol 112:133–141

Adams MJ, Jones DR, O'Neill TM, Hill SA (1993) The effect of cereal break crops on barley mild mosaic virus. Ann Appl Biol 123:37–45

Anonymous (2011) Polymyxa graminis. In: Distribution maps of plant diseases no 1108. Edited by CABI/EPPO, Wallingford, UK

Baltenberger DE, Ohm HW, Foster JE (1987) Reactions of oat, barley, and wheat to infection with barley yellow dwarf virus isolates. Crop Sci 27:195–198

Bauer E, Weyen J, Schiemann A, Graner A, Ordon F (1997) Molecular mapping of novel resistance genes against barley mild mosaic virus (BaMMV). Theor Appl Genet 95:1263–1269

Bukvayová N, Henselová M, Vajciková V, Kormanová T (2006) Occurrence of dwarf virus of winter wheat and barley in several regions of Slovakia during the growing seasons 2001–2004. Plant Soil Environ 52:392–401

Catherall PL, Jones AT, Hayes JD (1970) Inheritance and effectiveness of genes in barley that condition tolerance to barley yellow dwarf virus. Ann Appl Biol 65:153–161

Chalhoub BA, Sarrafi A, Lapierre HD (1995) Partial resistance in the barley (Hordeum vulgare L) cultivar 'Chikurin-Ibaraki-1' to two PAV-like isolates of barley yellow-dwarf virus: allelic variability at the Yd2 gene locus. Plant Breed 114:303–307

Chen J, Swaby AG, Adams MJ, Ruan Y (1991) Barley mild mosaic virus inside its fungal vector, Polymyxa graminis. Ann Appl Biol 118:615–621

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L et al (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582

Collins NC, Paltridge NG, Ford CM, Symons RH (1996) The Yd2 gene for barley yellow dwarf virus resistance maps close to the centromere on the long arm of barley chromosome 3. Theor Appl Genet 92:858–864

Comadran J, Kilian B, Russell J, Ramsay L, Stein N, Ganal M, Shaw P, Bayer M, Thomas W, Marshall D, Hedley P, Tondelli A, Pecchioni N, Francia E, Korzun V, Walther A, Waugh R (2012) A homologue of Antirrhinum CENTRORADIALIS is a component of the quantitative photoperiod and vernalization independent EARLINESS PER SE 2 locus in cultivated barley. Nat Genet 44:1388–1392

Davidson AD, Prols M, Schell J, Steinbiss HH (1991) The nucleotide-sequence of RNA 2 of barley yellow mosaic virus. J Gen Virol 72:989–993

Dessens JT, Nguyen M, Meyer M (1995) Primary structure and sequence analysis of RNA2 of a mechanically transmitted barley mild mosaic virus isolate: an evolutionary between bymo- and furoviruses. Arch Virol 140:325–333

Diaz-Pendon JA, Truniger V, Nieto C, Garcia-Mas J, Bendahmane A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5:223–233

Edwards MC, Steffenson BJ (1996) Genetics and mapping of barley stripe mosaic virus resistance in barley. Phytopathology 86:184–187

Ford CM, Paltridge NG, Rathjen JP, Moritz RL, Simpson RJ, Symons RH (1998) Rapid and informative assays for Yd2, the barley yellow dwarf virus resistance gene, based on the nucleotide sequence of a closely linked gene. Mol Breed 4:23–31

Friedt W, Ordon F (2007) Molecular markers for gene pyramiding and resistance breeding in barley. In: Varshney R, Tuberosa R (eds) Genomics-assisted crop improvement, vol 2, Genomics applications in crops. Springer, Heidelberg, pp 81–101

Götz R, Friedt W (1993) Resistance to the barley yellow mosaic-virus complex – differential genotypic reactions and genetics of BaMMV resistance of barley (Hordeum vulgare L.). Plant Breed 111:125–131

Graner A, Bauer E (1993) RFLP mapping of the ym4 virus resistance gene in barley. Theor Appl Genet 86:689–693

Graner A, Bauer E, Kellermann A, Proeseler G, Wenzel G, Ordon F (1995) RFLP analysis of resistance to the barley yellow mosaic virus complex. Agronomie 15:475–479

Graner A, Bauer E, Chojecki J, Tekauz A, Kellermann A, Proeseler G, Michel M, Valkov V, Wenzel G, Ordon F (1996) Molecular mapping of disease resistance in barley. In: Scoles G, Rossnagel B (eds) Proceedings of 5th international oat conference and 7th international barley genetics symposium poster sessions, vol 1. University Extension Press, Saskatoon, Sakatchewan, pp 253–255

Graner A, Streng S, Kellermann A, Proeseler G, Schiemann A, Peterka H, Ordon F (1999a) Molecular mapping of genes conferring resistance to soil-borne viruses in barley – an approach to promote understanding of host-pathogen interactions. J Plant Dis Protect 106:405–410

Graner A, Streng S, Kellermann A, Schiemann A, Bauer E, Waugh R, Pellio B, Ordon F (1999b) Molecular mapping and genetic fine-structure of the rym5 locus encoding resistance to different strains of the barley yellow mosaic virus complex. Theor Appl Genet 98:285–290

Habekuss A, Kuhne T, Kramer I, Rabenstein F, Ehrig F, Ruge-Wehling B, Huth W, Ordon F (2008a) Identification of barley mild mosaic virus isolates in Germany breaking rym5 resistance. J Phytopathol 156:36–41

Habekuss A, Riedel C, Schliephake E, Ordon F (2008b) Breeding for resistance to insect transmitted viruses in barley – an emerging challenge due to global warming. J Cultivated Plants 61:87–91

Hofinger BJ, Russel JR, Bass CG, Baldwin T, dos Reis M, Hedley PE, Li Y, Macaulay M, Waugh R, Hammond-Kosack KE, Kanyuka K (2011) An exceptionally high nucleotide and haplotype diversity and a signature of positive selection for the eIF4E resistance gene in barley are revealed by allele mining and phylogenetic analyses of natural populations. Mol Ecol 17:3653–3668

Humbroich K, Jaiser H, Schiemann A, Devaux P, Jacobi A, Cselenyi L, Habekuss A, Friedt W, Ordon F (2010) Mapping of resistance against Barley mild mosaic virus-Teik (BaMMV) – an rym5 resistance breaking strain of BaMMV – in the Taiwanese barley (Hordeum vulgare) cultivar 'Taihoku A'. Plant Breed 129:346–348

Huth W (1988) Ein Jahrzehnt Barley yellow mosaic virus in der Bundesrepublik Deutschland. Nachrichtenbl Dt Pflanzenschutzd 40:49–55

Huth W, Adams MJ (1990) Barley yellow mosaic virus (BaYMV) and BaYMV-M: two different viruses. Intervirology 31:38–42

Huth W, Lesemann DE (1978) Eine für die Bundesrepublik neue Virose an Wintergerste. Nachrichtenbl Dt Pflanzenschutzd 30:184–185

Huth W, Lesemann DE, Paul HL (1984) Barley yellow mosaic virus: purification, electron microscopy, serology and other properties of two types of the virus. Phytopathol Z 111:37–54

Ikata S, Kawai I (1940) Studies on wheat yellow mosaic disease. Noji Kairyo Shiro 154:1–123 (zit Inouye & Saito 1975)

International Barley Genome Sequencing Consortium (IBSC) (2012) A physical, genetical and functional sequence assembly of the barley genome. Nature 491:711–716

Kai H, Takata K, Tsukazaki M, Furusho M, Bana T (2012) Molecular mapping of Rym17, a dominant and rym18 a recessive barley yellow mosaic virus (BaYMV) resistance genes derived from Hordeum vulgare L. Theor Appl Genet 124:577–583

Kanyuka K, Ward E, Adams MJ (2003) Polymyxa graminis and the cereal viruses it transmits: a research challenge. Mol Plant Pathol 4:393–406

Kanyuka K, McGrann G, Alhudaib K, Hariri D, Adams MJ (2004) Biological and sequence analysis of a novel European isolate of Barley mild mosaic virus that overcomes the barley rym5 resistance gene. Arch Virol 149:1469–1480

Kanyuka K, Druka A, Caldwell DG, Tymon A, McCallum N, Waugh R, Adams MJ (2005) Evidence that the recessive bymovirus resistance locus rym4 in barley corresponds to the eukaryotic translation initiation factor 4E gene. Mol Plant Pathol 6:449–458

Kashiwazaki S (1996) The complete nucleotide sequence and genome organization of barley mild mosaic virus (Na1 strain). Arch Virol 141:2077–2089

Kashiwazaki S, Ogawa K, Usugi T, Omura T, Tsuchizaki T (1989) Characterization of several strains of barley yellow mosaic virus. Ann Phytopathol Soc Jpn 55:16–25

Kashiwazaki S, Minobe Y, Omura T, Hibino H (1990) Nucleotide sequence of barley yellow mosaic virus RNA1: a close evolutionary relationship with potyviruses. J Gen Virol 71:2781–2790

Kashiwazaki S, Minobe Y, Hibino H (1991) Nucleotide sequence of barley yellow mosaic virus RNA2. J Gen Virol 72:995–999

Kraakman ATW, Martinez F, Mussiraliev B, van Eeuwijk FA, Niks RE (2006) Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars. Mol Breed 17:41–58

Kühne T (2009) Soil-borne viruses affecting cereals – known for long but still a threat. Virus Res 141:174–183

Kühne T, Shi N, Proeseler G, Adams MJ, Kanyuka K (2003) The ability of a bymovirus to overcome the rym4-mediated resistance in barley correlates with a codon change in the VPg coding region on RNA1. J Gen Virol 84:2853–2859

Le Gouis J, Devaux P, Werner K, Hariri D, Bahrman N, Beghin D, Ordon F (2004) Rym15 from the Japanese culrivar 'Chikurin Ibaraki 1' is a new barley mild mosaic virus (BaMMV) resistance gene mapped on chromosome 6H. Theor Appl Genet 108:1521–1525

Legreve A, Delfosse P, Maraite H (2002) Phylogenetic analysis of Polymyxa species based on nuclear 5.8S and internal transcribed spacers ribosomal DNA sequences. Mycol Res 106:138–147

Leonard S, Plante D, Wittmann S, Daigneault N, Fortin MG, Laliberte JF (2000) Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. J Virol 74:7730–7737

Lindsten K, Vacke J (1991) A possible barley adapted strain of wheat dwarf virus (Wdv). Acta Phytopathol Entomol Hung 26:175–180

Lister RM, Ranieri R (1995) Distribution and economic importance of barley yellow dwarf. In: D'Arcy CJ, Burnett PA (eds) Barley yellow dwarf: 40 years of progress. APS, St. Paul, MN, pp 29–53

Luepken T, Stein N, Perovic D, Habekuß A, Krämer I, Hähnel U, Steuernagel B, Scholz U, Zhou R, Ariyadasa R, Taudien S, Platzer M, Martis M, Mayer K, Friedt W, Ordon F (2013) Genomics-based high-resolution mapping of the BaMMV/BaYMV resistance gene rym11 in barley (Hordeum vulgare L.). Theor Appl Genet 126:1201–1212

Maule AJ, Caranta C, Boulton MI (2007) Sources of natural resistance to plant viruses: status and prospects. Mol Plant Pathol 8:223–231

Mayer KFX, Martis M, Hedley PE, Kimková H, Liu H, Morris JA, Steuernagel B, Taudien S, Roessner S, Gundlach H, Kubaláková M, Suchánková P, Murat F, Felder M, Nussbaumer T, Graner A, Salse J, Endo T, Sakai H, Tanaka T, Itoh T, Sato K, Platzer M, Matsumoto T, Scholz U, Doleqel J, Waugh R, Stein N (2011) Unlocking the barley genome by chromosomal and comparative genomics. Plant Cell 23:1249–1263

Meyer M, Dessens JT (1996) The complete nucleotide sequence of barley mild mosaic virus RNA1 and its relationship with other members of the Potyviridae. Virology 219:268–273

Michon T, Estevez Y, Walter J, German-Retana S, Le Gall O (2006) The potyviral virus genome-linked protein VPg forms a ternary complex with the eukaryotic initiation factors eIF4E and eIF4G and reduces eIF4E affinity for a mRNA cap analogue. FEBS J 273:1312–1322

Miller WA, Rasochova L (1997) Barley yellow dwarf viruses. Annu Rev Phytopathol 35:167–190

Monzingo AF, Dhaliwal S, Dutt-Chaudhuri A, Lyon A, Sadow JH, Hoffman DW, Robertus JD, Browning KS (2007) The structure of eukaryotic translation initiation factor-4E from wheat reveals a novel disulfide bond. Plant Physiol 143:1504–1518

Niks RE, Habekuß A, Bekele B, Ordon F (2004) A novel major gene on chromosome 6H for resistance to barley against the barley yellow dwarf virus. Theor Appl Genet 109:1536–1543

Nissan-Azzous F, Graner A, Friedt W, Ordon F (2005) Fine-mapping of the BaMMV, BaYMV-1 and BaYMV-2 resistance of barley (Hordeum vulgare) accession PI1963. Theor Appl Genet 110:212–218

Okada Y, Kanatani R, Arai S, Ito K (2004) Interaction between barley yellow mosaic disease-resistance gene rym1 and rym5, in the response to BaYMV strains. Breed Sci 54:319–325

Ordon F (2009) Coordinator's report: disease and pest resistance genes. Barley Genet Newsl 39:58–67

Ordon F, Friedt W (1993) Mode of inheritance and genetic diversity of BaMMV-resistance of exotic barley germplasms carrying genes different from 'ym4'. Theor Appl Genet 86:229–233

Ordon F, Friedt W (1994) Agronomic traits of exotic barley germplasms resistant to soil-borne mosaic-inducing viruses. Genet Crop Evol 41:43–46

Ordon F, Götz R, Friedt W (1993) Genetic stocks resistant to barley yellow mosaic viruses (BaMMV, BaYMV, BaYMV-2) in Germany. Barley Genet Newsl 22:44–48

Ordon F, Bauer E, Friedt W, Graner A (1995) Marker-based selection for the ym4 BaMMV-resistance gene in barley using RAPDs. Agronomie 15:481–485

Ordon F, Pellio B, Werner K, Schiemann A, Friedt W, Graner A (2003) Molecular breeding for resistance to soil-borne viruses (BaMMV, BaYMV, BaYMV-2) of barley (Hordeum vulgare L.). J Plant Dis Protect 110:287–295

Ordon F, Habekuss A, Kastirr U, Rabenstein F, Kühne T (2009) Virus resistance in cereals: sources of resistance, genetics and breeding. J Phytopathol 157:535–554

Palloix A, Ordon F (2011) Advanced breeding for virus resistance in plants. In: Caranta C, Miguel A, Aranda MA, Tepfer M, López-Moya JJ (eds) Recent advances in plant virology. Caister Academic, Norfolk, pp 195–218

Paltridge NG, Collins NC, Bendahmane A, Symons RH (1998) Development of YLM, a codominant PCR marker closely linked to the Yd2 gene for resistance to barley yellow dwarf disease. Theor Appl Genet 96:1170–1177

Peerenboom E, Pröls M, Schell J, Steinbiß HH, Davidson AD (1992) The complete nucleotide sequence of RNA1 of a German isolate of barley yellow mosaic virus and its comparison with a Japanese isolate. J Gen Virol 73:1303–1308

Pellio B, Streng S, Bauer E, Stein N, Perovic D, Schiemann A, Friedt W, Ordon F, Graner A (2005) High-resolution mapping of the Rym4/Rym5 locus conferring resistance to the barley yellow mosaic virus complex (BaMMV, BaYMV, BaYMV-2) in barley (Hordeum vulgare ssp. vulgare L.). Theor Appl Genet 110:283–293

Plumb RT, Lennon EA, Gutterridge RA (1986) The effects of infection by barley yellow mosaic virus on the yield and components of yield of barley. Plant Pathol 35:314–318

Prins M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M (2008) Strategies for antiviral resistance in transgenic plants. Mol Plant Pathol 9:73–83

Ramsell J, Boulton MI, Martin D, Valkonen J, Kvarnheden A (2009) Studies on the host range of the barley strain of Wheat dwarf virus using an agroinfectious viral clone. Plant Pathol 58:1161–1169

Rhoads RE (2009) eIF4E: new family members, new binding partners, new roles. J Biol Chem 284:16711–16715

Riedel C, Habekuss A, Schliephake E, Niks R, Broer I, Ordon F (2011) Pyramiding of Ryd2 and Ryd3 conferring tolerance to a German isolate of Barley yellow dwarf virus (BYDV-PAV-ASL-1) leads to quantitative resistance against this isolate. Theor Appl Genet 123:69–76

Ruge B, Linz A, Proeseler G, Pickering G, Greif P, Wehling P (2003) Mapping of Rym14Hb, a gene introgressed from Hordeum bulbosum and conferring resistance to BaMMV and BaYMV in barley. Theor Appl Genet 107:965–971

Ruge-Wehling B, Linz A, Habekuß A, Wehling P (2006) Mapping of Rym16 Hb, the second soil-borne virus-resistance gene introgressed from Hordeum bulbosum. Theor Appl Genet 113:867–873

Saeki K, Miyazaki C, Hirota N, Saito A, Ito K, Konishi T (1999) RFLP mapping of BaYMV resistance gene rym3 in barley (Hordeum vulgare). Theor Appl Genet 99:727–732

Sato K, Nankaku N, Takeda K (2009) A high-density transcript linkage map of barley derived from a single population. Heredity 103:110–117
Schaller CW (1984) The genetics of resistance to barley yellow dwarf virus in barley. In: Burnett PA (ed) Barley yellow dwarf. Proceedings of a workshop. CIMMYT, Mexico DF, p 93
Schaller CW, Qualset CO, Rutger JN (1964) Inheritance and linkage of the Yd2 gene conditioning resistance to the barley yellow dwarf disease in barley. Crop Sci 4:544–548
Scheurer KS, Huth W, Friedt W, Ordon F (2000) First results on BYDV-tolerance in barley estimated in pot experiments. J Plant Dis Prot 107:427–432
Scheurer KS, Friedt W, Huth W, Waugh R, Ordon F (2001) QTL analysis of tolerance to a German strain of BYDV-PAV in barley (Hordeum vulgare L.). Theor Appl Genet 103:1074–1083
Scholz M, Ruge-Wehling B, Habekuss A, Schrader O, Pendinen G, Fischer K, Wehling P (2009) Ryd4Hb: a novel resistance gene introgressed from Hordeum bulbosum into barley and conferring complete and dominant resistance to the barley yellow dwarf virus. Theor Appl Genet 119:837–849
Schubert J, Habekuss A, Kazmaier K, Jeske H (2007) Surveying cereal-infecting geminiviruses in Germany – diagnostics and direct sequencing using rolling circle amplification. Virus Res 127:61–70
Shukla VK, Doyon Y, Miller JC et al (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature 459:437–443
Sip V, Sirlova L, Chrpova J (2006) Screening for barley yellow dwarf virus-resistant barley genotypes by assessment of virus content in inoculated seedlings. J Phytopathol 154:336–342
Skaria M, Lister RM, Foster JE, Shaner G (1985) Virus content as an index of symptomatic resistance to barley yellow dwarf virus in cereals. Phytopathology 75:212–216
Sotome T, Kawada N, Kato T, Sekiwa T, Nishigawa H, Natsuaki T, Kimura K, Maeoka Y, Nagamine T, Kobayashi S, Wada Y, Yoshida T (2010) The current and new strains of barley yellow mosaic virus (BaYMV) in Tochigi prefecture. Jpn J Crop Sci 79:29–36
Stein N, Perovic D, Kumlehn J, Pellio B, Stracke S, Streng S, Ordon F, Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive bymovirus resistance in Hordeum vulgare (L.). Plant J 42:912–922
Stracke S, Presterl T, Stein N, Perovic D, Ordon F, Graner A (2007) Effects of introgression and recombination on haplotype structure and linkage disequilibrium surrounding a locus encoding Bymovirus resistance in barley. Genetics 175:805–817
Subr Z, Fomitcheva VW, Kühne T (2000) The complete sequence of RNA1 of the German isolate BaMMV-ASL1 and preparation of an antiserum specific to the nonstructural protein P3. J Phytopathol 148:461–467
Sun W, Chen J, Wang L, Fu T (2008) Construction of twin T-DNA vector encoding hairpin RNA targeting the replicase gene of barley yellow dwarf virus. Chin J Appl Environ Biol 14:745–749
Suneson CA (1955) Breeding for resistance to yellow dwarf virus in barley. Agron J 47:283
Takahashi R, Hayashi J, Moriya I, Hirao C (1970) Studies on resistance to yellow mosaic disease in barley. III. Inheritance and linkage studies of the resistance. Nogaku Kenyu 53:153–165
Thompson JP, Clewett TG, Jennings RE, Sheedy JG, Owen KJ, Persley DM (2011) Detection of Polymyxa graminis in a barley crop in Australia. Aust Plant Pathol 40:66–75
Timpe U, Kühne T (1994) The complete nucleotide sequence of RNA 2 of Barley mild mosaic virus (BaMMV). Eur J Plant Pathol 100:233–242
Toojinda T, Broers LH, Chen XM, Hayes PM, Kleinhofs A, Korte J, Kudrna D, Leung H, Line RF, Powell W, Ramsay L, Vivar H, Waugh R (2000) Mapping quantitative and qualitative disease resistance genes in a doubled haploid population of barley (Hordeum vulgare). Theor Appl Genet 101:580–589
Tyrka M, Perovic D, Wardynska A, Ordon F (2008) A new diagnostic SSR marker for selection of the Rym4/Rym5 locus in barley breeding. J Appl Genet 49:127–134

Uptmoor R, Wenzel W, Ayisi K, Donaldson G, Gehringer A, Friedt W, Ordon F (2006) Variation of the genomic portion of the recurrent parent in BC1 and its relation to yield performance in sorghum (Sorghum bicolor) breeding for low-input conditions. Plant Breed 125:532–534

Vacke J (1961) Wheat dwarf virus disease. Biol Plant 3:228

Vacke J, Cibulka R (2001) Reaction of registered winter barley varieties to Wheat dwarf virus infection. Czech J Genet Plant Breed 37:50–52

Vaianopoulos C, Bragard C, Moreau V, Maraite H, Legreve A (2007a) Identification and quantification of Polymyxa graminis f. sp temperata and P. graminis f. sp tepida on barley and wheat. Plant Dis 91:857–864

Vaianopoulos C, Legreve A, Moreau V, Steyer S, Maraite H, Bragard C (2007b) Barley yellow mosaic virus is overcoming rym4 resistance in Belgium. Commun Agric Appl Biol Sci 72:333–339

Wellie-Stephan O, Schäfer BC, Rabenstein F, Kühne T (2010) BaYMV-2 regional stark verbreitet. Ergebnisse des bundesweiten Monitorings zum Gelbmosaikvirus Typ 2 2009 und 2010. Innovation 4:18–20

Werner K, Friedt W, Ordon F (2000) Strategies for pyramiding resistance genes against the barley yellow mosaic virus complex based on molecular markers and DH-lines. Proceedings of 8th international barley genetics symposium, vol 2, Adelaide, Australia, pp 200–202 (contributed papers)

Werner K, Friedt W, Laubach E, Waugh R, Ordon F (2003a) Dissection of resistance to soil-borne yellow mosaic inducing viruses of barley (BaMMV, BaYMV, BaYMV-2) in a complex breeders cross by SSRs and simultaneous mapping of BaYMV/BaYMV-2 resistance of 'Chikurin Ibaraki 1'. Theor Appl Genet 106:1425–1432

Werner K, Rönnicke S, Le Gouis J, Friedt W, Ordon F (2003b) Mapping of a new BaMMV-resistance gene derived from the variety 'Taihoku A'. J Plant Dis Protect 110:304–311

Werner K, Friedt W, Ordon F (2005) Strategies for pyramiding resistance genes against the barley yellow mosaic virus complex (BaMMV, BaYMV, BaYMV-2). Mol Breed 16:45–55

Weyen J, Bauer E, Graner A, Friedt W, Ordon F (1996) RAPD mapping of the distal portion of chromosome 3 of barley, including BAMMV/BaYMV resistance gene ym4. Plant Breed 115:285–287

Wittmann S, Chatel H, Fortin MG, Laliberte JF (1997) Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of Arabidopsis thaliana using the yeast two-hybrid system. Virology 234:84–92

Yang P, Lüpken T, Habekuß A, Hensel G, Steuernagel B, Kilian B, Aiyadasa R, Himmelbach A, Kumlehn J, Scholz U, Ordon F, Stein N (2014) Protein disulfide isomerase like 5-1 is a susceptibility factor to plant viruses. Proc Natl Acad Sci USA 111:2104–2109

Zhang Y, Zhang F, Li XH, Baller JA, Qi YP, Starker CG, Bogdanove AJ, Voytas DF (2013) Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol 161:20–27

Chapter 11
Host and Nonhost Response to Attack by Fungal Pathogens

Patrick Schweizer

11.1 Introduction

Barley is attacked by a large number of fungal pathogens to most of which it responds as a resistant nonhost. A relatively small subset of adapted fungi successfully infect barley and of those, an even smaller subset cause agronomically important fungal diseases. Most important fungal diseases are leaf and stem rust caused by *Puccinia hordei* (Fig. 11.1b) and *Puccinia graminis* f.sp. *tritici*, respectively; powdery mildew caused by *Blumeria graminis* f.sp. *hordei* (Fig. 11.1a); scald caused by *Rhynchosporium commune* previously designated *R. secalis* (Fig. 11.1g); net-type and spot-type leaf blotch caused by *Pyrenophora teres* f. *teres* (Fig. 11.1d) and f. *maculata*, respectively; spot blotch caused by *Bipolaris sorokiniana* (Fig. 11.1e); head blight caused by *Fusarium graminearum* and *F. culmorum* (Fig. 11.1f) and leaf blast caused by *Magnaporthe oryzae* (Fig. 11.1c), a rice pathogen that also more and more endangers wheat grown in Brazil in crop rotation with rice (Urashima et al. 1993). Barley is another host although its cultivation is not yet seriously affected by *M. oryzae*, a situation that might change soon in the light of global warming that is expected to push plant disease borders towards higher northern and southern latitudes. The above-mentioned pathogens will be in the focus of this review, while the increasingly serious leaf spot disease caused by *Ramularia collo-cygni* will only be addressed briefly here due to virtually still lacking information related to plant responses (Fig. 11.1). All these fungi belong to the *Ascomycetes* except *Puccinia* sp. that belongs to the *Basidiomycetes*. In the case of *Ramularia collo-cygni*, for which no teleomorph form has been described so far, a fruiting body-based classification is not possible. Except for *Fusarium* sp. they all cause leaf diseases, which opens up

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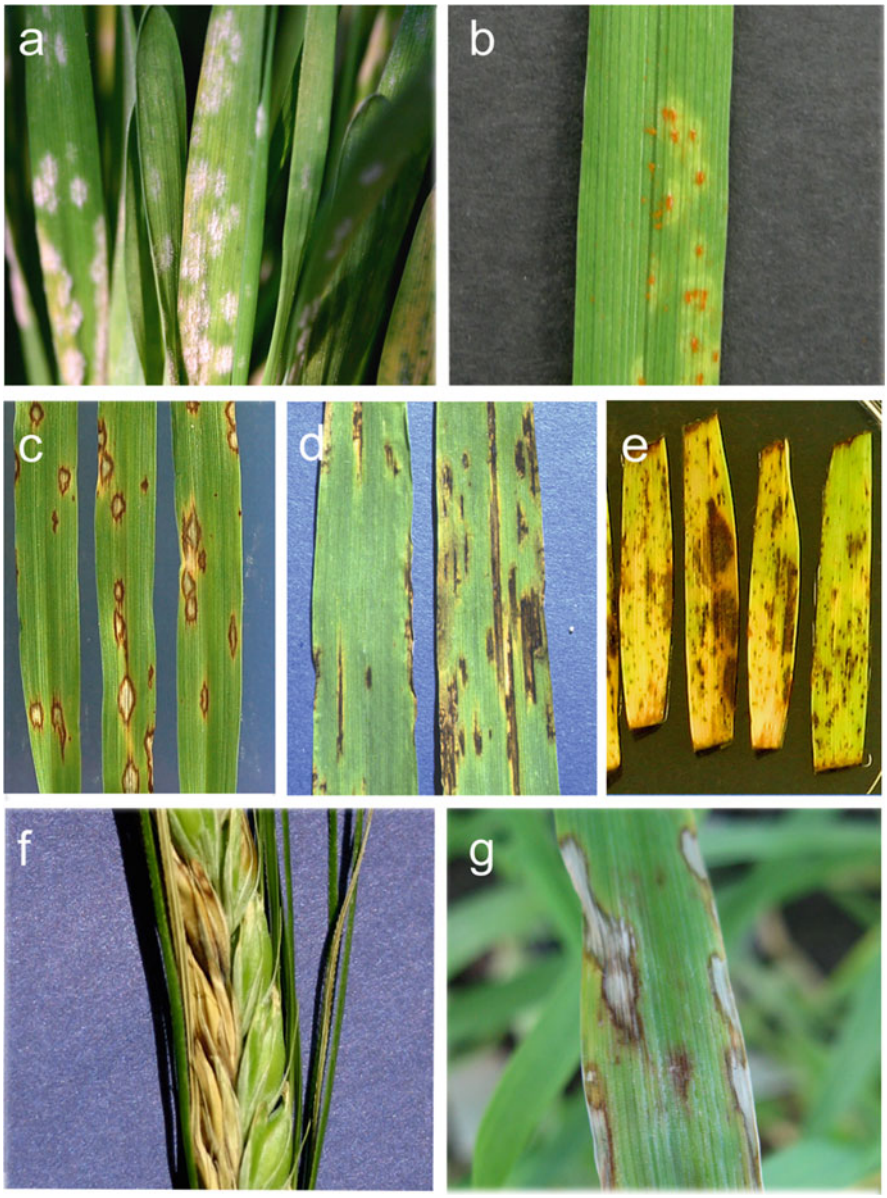

Fig. 11.1 Disease symptoms caused by seven important barley fungal pathogens. (**a**) *Blumeria graminis* f.sp. *hordei* causing powdery mildew. (**b**) *Puccinia hordei* causing leaf rust, (**c**) *Magnaporthe oryzae* causing leaf blast. (**d**) *Pyrenophora teres* f. *teres* causing net-type leaf blotch. (**e**) *Bipolaris sorokiniana* causing spot blotch. (**f**) *Fusarium graminearum* and *F. culmorum* causing head blight. (**g**) *Rhynchosporium commune* (previously: *R. secalis*) causing scald

the possibility to directly compare barley responses in this organ to the different fungi (Table 11.1). In most cases the leaf epidermis is the first tissue to be penetrated by mostly asexual spores, and this commonality puts forward barley responses in the epidermis as outstandingly important for the success or failure of the individual fungal attacks. The seven pathogens of interest here can be placed along a gradient of different life styles ranging from obligate biotrophic (*B. graminis* and *Puccinia* sp.) over hemibiotrophic (*B. sorokiniana*, *P. teres*, *M. oryzae*) to necrotrophic (*R. commune* and *Fusarium* sp.). Obligate biotrophic pathogens can only exist on living host tissue and are therefore entirely dependent on constant support by the host plant. By contrast, necrotrophic pathogens secrete toxins and shrive on dying or dead plant material. Lastly, hemibiotrophic pathogens start softly by leaving host cells alive and switch usually 1–3 days after initial infection to the more brute-force approach by killing invaded host tissue via toxins or removal of cell death suppressors (effectors) thereby provoking host cell suicide as a co-opted defence reaction [for a review see Horbach et al. (2011)].

It has been shown that barley responds to these fungal pathogens with altered gene expression often leading to the accumulation of pathogenesis-related (PR) proteins, with cell-wall appositions and sometimes with local cell death responses known as hypersensitive response (HR) (Thordal-Christensen et al. 2000; Kumar et al. 2002; Niks and Rubiales 2002; Steiner-Lange et al. 2003; Jarosch et al. 2005; Boddu et al. 2006; Huckelhoven 2007; Huckelhoven and Panstruga 2011; Linsell et al. 2011; Liu et al. 2011). However, we cannot a priori assume a uniform response of the attacked plant to the different fungi because each of them probably achieved its own way of host adaptation as a result of host–pathogen co-evolution over millions of years. A classical example in this respect is the clearly distinct defence responses triggered in the model plant *Arabidopsis thaliana* in response to biotrophic versus necrotrophic pathogens, the former primarily mediated by the stress hormone salicylic acid, whereas the latter is orchestrated by combined jasmonate and ethylene signalling (Glazebrook 2005). In the light of these findings, it appears more likely that all fungal barley diseases represent distinct cases of plant–microbe interactions as reflected by clearly distinct infection strategies, morphological infection structures, infected tissue types, etc. Moreover, looking at a host plant as kind of small-scale ecosystem for parasites, it might even be expected that coexisting pathogens occupy each a specific host niche not too much affected by competition for resources by other pathogens or by plant responses to any co-infecting pathogen. As a consequence, different host susceptibility factors might have become co-opted for establishing successful infection, and different host defence genes and components might be relevant for warding off the attack or limiting fungal spread.

Ultimately, the presence or absence of strong resistance genes, different efficiencies of host factors to limit fungal infection and different efficiencies of co-opting host susceptibility factors determine the severity of an infection. For each of the barley–fungal interactions in the focus of this article, quantitative or qualitative resistance has been described and can therefore be used for crop improvement by (pre-)breeding approaches. However, it is unclear at present if

Table 11.1 Fungal development in barley (asexual life cycle)

Fungus	Initial spore contact in	Epidermal cell penetration	Massive hyphal growth on/in	Biotrophic phase	Necrotrophic phase	Haustorium	Obligate biotroph
Blumeria graminis	Leaf/shoot epidermis	Yes	Leaf surface	Yes	No	Yes	Yes
Puccinia sp.	Leaf/shoot epidermis	No	Leaf mesophyll	Yes	No	Yes	Yes
Pyrenophora teres	Leaf epidermis	Yes	Leaf mesophyll	Yes	Yes	No	No
Bipolaris sorokiniana	Leaf epidermis	Yes	Leaf mesophyll	Yes	Yes	No	No
Magnaporthe oryzae	Leaf epidermis	Yes	Leaf mesophyll	Yes	Yes	No	No
Rhynchosporium comm.	Leaf epidermis	No	Leaf epidermis	No	Yes	No	No
Fusarium sp.	Floral bract epidermis	Yes	Ovary, rachis	Unclear	Yes	No	No

the many identified and mapped major resistance (R) or quantitative trait loci (QTL) for resistance share common genes. In the worst case, stacking of such loci will have to be achieved separately for all major fungal diseases present at a given agroecological system. What might be even worse is the considerable risk that the improvement of resistance to one disease has a trade-off for another one especially if they are caused by pathogens with different life styles. For example, improving on rust resistance by enhancing the potential of the plant to trigger hypersensitive cell death might be associated with enhanced susceptibility to nectotrophic pathogens such as *R. commune*-producing toxins that kill host cells. The same trade-off problem probably applies to the engineering of broadly acting fungal resistance into transgenic crops and might explain why so far mostly heterologous antifungal proteins and not host defence-regulating factors have been introduced (Collinge et al. 2010).

The aim of this review is to summarize our current knowledge of barley responses to its major fungal pathogens at the molecular, cellular and whole-plant level and to compare susceptibility as well as resistance mechanisms plus the genetic loci that affect these interactions. Unfortunately detailed knowledge about the plant's response to fungal attack under field conditions is still very limited although highly important, e.g. for identifying transgenic lead events for durable disease resistance. Therefore this aspect cannot be properly addressed at the moment. However, information collected in the lab, in growth chambers and in greenhouses can provide valuable first hints to plant pathologists, breeders and genetic engineers for the selection of more promising approaches to cope with the immense disease problem impacting on the identified goal to secure and stabilize yield under conditions of climatic change and decreasing productive acreage per capita of the world population.

11.2 Response to Biotrophic Pathogens

Barley is a host to three obligate biotrophic pathogens: *Blumeria graminis* f.sp. *hordei* (*Bgh*) causing powdery mildew and *Puccinia hordei* (*Ph*) and *Puccinina graminis* f.sp. *tritici* (*Pgt*) causing leaf and stem rust, respectively. Being obligate biotrophic organisms they cannot grow on dead plant material or artificial media, which makes them quite intractable for functional genomics or genetic approaches. Powdery mildew and rust are very widespread diseases, causing potential problems basically everywhere where barley is grown. Without control by plant resistance genes or fungicides, they often cause moderate to severe yield reduction although they are not devastating (Lim and Gaunt 1986). Two important aspects of their success are (1) the enormous epidemiological potential due to massive production of asexual spores that can be transported by wind over distances of hundreds of kilometres and (2) an intact sexual life cycle rendering them capable of combining beneficial alleles and rapidly adapting to challenges opposed, e.g. by the introduction of new R-genes (McDonald and Linde 2002). Although *Bgh*, *Ph* and *Pgt* belong to the phylogenetically distant groups of the *Ascomycetes* and

Basidiomycetes, respectively, they share some basic mechanisms of how to successfully invade barley: First, all three fungi—after having penetrated the epidermal (*Bgh*) or substomatal (*Ph* and *Pgt*) cell wall by a penetration peg emerging from the first appressorium (*Bgh*) or from the haustorial mother cell (*Ph* and *Pgt*)—form a haustorium that is specialized in nutrient uptake from the host and delivery to the growing fungal colony. Second, this haustorium only invaginates the host plasmalemma and thus remains an apoplastic structure. Third, neither switches to a necrotrophic phase under any condition, which is reflected by the absence of host cell death in a fully susceptible interaction (Niks and Rubiales 2002; Huckelhoven and Panstruga 2011).

11.2.1 Blumeria graminis

The interaction of barley with *Bgh* as well as the wheat powdery mildew *B. graminis* f.sp. *tritici* (*Bgt*) represents probably the best examined pathosystem in cereals, followed by the wheat/*Fusarium* head blight and the rice/*M. oryzae* interactions. In fact a large amount of detailed descriptive and—since more recently—functional data is available characterizing this interaction, which therefore can be considered a plant–pathogen model. However, due to limited space and also for the sake of comparability of data to the other diseases to be addressed here, the discussion will be restricted to (1) parallel transcript profiling, (2) microscopic analysis of cellular changes in attacked cells including the formation of reactive oxygen species (ROS) and (3) gene function from transient, as well as stable silencing or overexpression approaches.

Barley rapidly recognizes the presence of powdery mildew spores on its leaf surface and responds by up-regulating a number of transcripts from about 3–6 h after inoculation (h.a.i.) onwards, irrespective of the final outcome of the interaction (Gregersen et al. 1997). This implies the rapid release and diffusion across the cuticle of pathogen-associated molecular patterns (PAMPs) and perception probably by pathogen-recognition receptors (PRRs). The transcriptome of powdery mildew-attacked barley has been studied both in whole leaf and peeled epidermal samples (Caldo et al. 2004, 2006; Moscou et al. 2011a; Zierold et al. 2005; Zellerhoff et al. 2010). Because the shoot epidermis is the only tissue in direct contact with the fungus, its response is highly relevant to the final outcome of the interaction. The transcript analysis revealed clear differences in the sets of regulated transcripts between the epidermis and entire leaves mostly reflecting differences between epidermal and the bulk of mesophyll cells. In the epidermis the transcriptional response was found to peak between 12 and 24 h.a.i., followed by a minimum at 48 h.a.i. and a massive reincrease from 72 h.a.i. onwards in the case of the susceptible interaction probably reflecting cellular and metabolic reorganization in order to support massive fungal growth on the leaf surface. Hand-curated, functional categorization of a good proportion of the barley transcriptome has been achieved by the MapMan tool and proved to be very useful for obtaining

information about pathways involved in biotic stress (Sreenivasulu et al. 2008). The late interaction was thus characterized by a distinct set of regulated transcripts not observed during the first 24 h.a.i. Outstanding in this respect were the functional categories of primary C- and N-metabolisms, which were strongly over-represented among late up-regulated transcripts (Metzner and Schweizer, unpublished). A direct comparison also revealed a stronger detectable, global response of the epidermis (ca. 8 % significantly regulated transcripts) compared to the entire leaf (ca. 5 % regulated transcripts). On the other hand, functional categories of regulated genes overlapped considerably between epidermis and entire leaves: In both tissues up-regulated genes encoding PR proteins as well as genes of redox regulation and amino acid metabolism were significantly over-represented, whereas downregulated genes of photosynthesis were strongly over-represented (Zellerhoff et al. 2010). As far as amino acid metabolism is concerned, an outstandingly strong and robust up-regulation of transcripts encoding enzymes for biosynthesis of tryptophan and phenylalanine was observed, both serving as precursors for defence-related compounds such as indole derivatives and lignin-like material (Caldo et al. 2004). In a comprehensive study, the effect of three alleles of the *Mla* R-gene on host transcriptome was examined (Moscou et al. 2011b). This led to the identification of 28 resistance-related transcripts with a remarkably broad range of predicted protein functions. The authors therefore concluded that *Mla* acts as positive regulator of a part of the host defence transcriptome. Among the downregulated transcripts, many encoding photosynthetic proteins were found in mesophyll but also in epidermal tissue. The latter observation was unexpected as barley epidermis is known to be photosynthetically inactive containing no chloroplasts except in guard cells. However, epidermal leucoplasts are the likely compartments of several plastidic biosynthetic pathways such as the shikimate or phenylpropanoid pathway, and light-independent roles of certain membrane-localized proteins of the photosynthetic machinery cannot a priori be excluded in this type of organelle.

At the microscopic level, attacked epidermal cells respond with the formation of cell-wall appositions at sites of attempted penetration, and the strength of this response tends to be correlated with resistance (Collinge 2009). Formation of efficient cell-wall appositions that are able to stop fungal penetration are associated with a massive focal reorganization of the cytoskeleton towards the point of attack by the penetration peg. Both microtubules and actin fibres were shown to become reorganized in this focal manner, and pharmacological as well as transient gene silencing and overexpression experiments support the view that they both are important for penetration resistance (Kobayashi et al. 1992; Miklis et al. 2007; Schmidt and Panstruga 2007; Huckelhoven and Panstruga 2011). In the case of penetration, cells either allow the formation of one or several *Bgh* haustoria or respond with hypersensitive cell death. It remains still open if this cell death response is reflecting apoptosis or autophagy or both (An et al. 2006). The fact that the barley bax inhibitor 1 (*BI-1*) gene encoding for a homologue of this well-characterized anti-apoptotic protein of animals suppresses *Bgh*-induced cell death and—upon overexpression—enhances susceptibility indicates that apoptotic cell

death is involved in the resistance response (Huckelhoven et al. 2003; Eichmann et al. 2010). Although no homologue of the pro-apoptotic *bax* gene has been identified in barley, it appears likely that an unknown barley gene fulfils this function. If the penetrated cell is not destined to hypersensitive cell death, it undergoes a series of morphological changes that are supposed to be enforced by fungal effectors: First, the focal reorganization of the cytoskeleton does not happen (Hoefle et al. 2011). Second, the membrane surrounding the growing haustorium extends in size and resembles a specific membrane microdomain (lipid raft) enriched in proteins such as the susceptibility mediator Mlo or the syntaxin Ror2 (Bhat et al. 2005). Haustorium accommodation by membrane reorganization was found to be dependent on host susceptibility factor HvRACB, a member of the small monomeric G-protein family, which is also known to be required for cell elongation (Schultheiss et al. 2005). Third, the HvRACB-interacting HvRIC171 protein was found to accumulate at sites of successful cell-wall penetration and enhanced susceptibility upon overexpression (Schultheiss et al. 2008). Strong evidence for the induction of lipid rafts by oomycete and fungal effectors has also been obtained in other plant systems (Kale et al. 2010).

As many other plants barley responds to powdery mildew attack with the accumulation of the ROS H_2O_2 and superoxide radical, detected in situ by diaminobenzidine (DAB) and nitrotetrazolium blue (NBT) staining, respectively. Generally the DAB-staining reaction was stronger in cells with penetration resistance reflecting stronger localized H_2O_2 accumulation at and around cell-wall appositions starting as early as 6 h.a.i. underneath primary germ tubes (Thordal-Christensen et al. 1997). In cells undergoing hypersensitive cell death, H_2O_2 accumulation was observed throughout the entire cell at 22 h.a.i. (Huckelhoven et al. 1999). Therefore, a local burst of hydrogen peroxide might be required for cross-linking reactions inside cell-wall appositions, whereas intracellular H_2O_2 accumulation might be a signal for triggering apoptosis. In the case of susceptible interactions, accumulation of superoxide radical was found in penetrated epidermal and underlying mesophyll cells indicating that formation of this oxygen radical is neither required for penetration-nor cell death-mediated resistance (Huckelhoven and Kogel 1998).

In recent years important functional information about barley genes that are relevant for the interaction with *B. graminis* was obtained by using transient assay systems. Two systems are currently in use: transient-induced gene silencing (TIGS) or transient overexpression by bombardment with DNA-coated gold particles and virus-induced gene silencing (VIGS) mediated by the barley stripe mosaic virus (BSMV). So far, between 10 and 100 barley genes have been functionally addressed in the literature, and more than 1,000 genes have been tested (Wise et al. 2009; Douchkov and Schweizer, unpublished). The body of published data suggested that genes involved in transcriptional regulation (Meng and Wise 2012), the generation or use of ROS such as *RBOHF2*, germin-like proteins or peroxidases (Zimmermann et al. 2006; Johrde and Schweizer 2008; Proels et al. 2010), genes involved in vesicle trafficking such as *Ror2* and *SNAP34* (Collins et al. 2003; Douchkov et al. 2005) and genes involved in signalling and cell death control

such as receptor-like kinases, transcription factors, G-proteins including interacting factors and Bax inhibitor 1 (Schultheiss et al. 2003; Jensen et al. 2007; Shen et al. 2007; Eichmann et al. 2010; Hoefle et al. 2011; Huesmann et al. 2012; Rayapuram et al. 2012) are of prime importance for different types of resistance or for supporting fungal growth. However, this list probably shows only the tip of an iceberg, and further genome-wide screens would be instrumental to obtain a more functional picture of the defence or susceptibility-related barley transcriptome. It should also be noted that results obtained from transient assays were confirmed in transgenic barley in several instances (see references cited above).

11.2.2 Puccinia hordei *and* P. graminis *f.sp.* tritici

Barley is a host of two rust species: *Puccinia hordei* (*Ph*) causing barley leaf rust and *Puccinia graminis* f.sp. *tritici* (*Pgt*) causing wheat and barley stem rust. The latter has become a major threat to global wheat and barley production due to the supervirulent Ug99 isolate first identified in 1999 in Uganda and since then spreading north and east towards Europe and (probably) India (Ward 2007). Despite their agronomic importance, *Ph* and *Pgt* have been less well studied compared to *Bgh*, and not until recently larger molecular datasets from rust interactions with barley have become publicly available. On the other hand interaction types and resistance mechanisms in barley have been accurately described at the cytological and genetic level (Niks 1983a, b; Marcel et al. 2007, 2008; Steffenson et al. 2009; Moscou et al. 2011a).

The transcriptome of rust-attacked barley has been analysed recently aiming at a general description of host changes as well as the identification of expression QTL (eQTL) loci for partial resistance (Chen et al. 2010b; Moscou et al. 2011b). Barley recognizes the presence of rust spores from approximately 12 h.a.i. onwards, while fungal development is still confined to the leaf or stem surface, before penetration of stomata by appressoria, and responds with the differential regulation of a large number of transcripts (Chen et al. 2010b; Zellerhoff et al. 2010). Over all analysed time points (12–48 h.a.i.), between 4 and 7 % of transcripts, were found to be significantly regulated. Functional classification among over 800 *Ph*-responsive transcripts revealed signalling components, transporters, stress-response proteins, transcription factors, PRRs and amino acid metabolism as the six most prominent functional categories.

Once the fungus has penetrated through the stomata, resistant barley genotypes mount a pre-haustorial defence response characterized by the formation of cell-wall appositions by cells of the substomatal cavity. Alternatively, these initially attacked cells or groups of neighbouring mesophyll cells trigger a controlled cell death programme (HR) (Niks 1983a, b). The type of cellular resistance response appears to be determined by the type of resistance genes or loci present: Penetration resistance at the cell wall was found to be associated with race-nonspecific, quantitative host-and nonhost resistance, whereas cell death was found to occur

in genotypes carrying major R-genes. Again, this situation is similar to that described for different barley–powdery mildew interactions. No information appears to be available with respect to the presence or absence of an oxidative burst in attacked cells, but it is likely that resistant barley would show similar responses as during *B. graminis* interactions because transcripts for ROS-generating enzymes such as peroxidases, oxalate oxidase (germin) and germin-like proteins were found to be upregulated in rust-attacked barley (Zellerhoff et al. 2010). Moreover, in resistant but not susceptible wheat/stem rust interactions, ROS accumulated in mesophyll cells (Wang et al. 2010).

VIGS by BSMV proved to be an interesting option for rapid functional assessment of (few) selected candidate genes affecting barley–rust interactions although this system has a lower throughput compared to the TIGS system described above in the barley–powdery mildew system. However, the TIGS system is limited to epidermal cells and therefore not suitable for addressing gene function in barley–rust interactions occurring mostly in the mesophyll. So far, genes encoding the NecS1 cell death suppressor and the Rpg5 R-protein were successfully tested using VIGS (Brueggeman et al. 2008; Zhang et al. 2009).

11.3 Response to Hemibiotrophic Pathogens

The interaction of hemibiotrophic fungal pathogens with barley or other hosts is characterized by a first phase of usually 24–48 h in which the pathogen avoids host cell death, probably also by actively suppressing it, and establishes initial, slowly growing hyphal structures inside or in close contact with the first attacked cells. Often the leaf epidermis is the tissue where this first, biotrophic phase takes place. Later, a switch to necrotrophic growth is observed, which is characterized by a change in hyphal morphology from thick and irregular to thin, regular and fast growing (Horbach et al. 2011). Such necrotrophic hyphae typically penetrate deeper into host tissues and ramify in the mesophyll. During the necrotrophic phase, fungal toxic metabolites or peptides such as prehelminthosporol (*B. sorokiniana*), toxin A to C (*P. teres*) and pyriculol and pyriculariol (*M. oryzae*) may be released in order to facilitate nutrient uptake from killed and disintegrated host cells. Disease caused by hemibiotrophic as well as necrotrophic pathogens is usually accompanied by necrotic lesions of few mm up to more than 1 cm in diameter or length, in contrast to the ones caused by the biotrophic pathogens *Bgh*, *Ph* and *Pt* that are characterized by visible spore production but only mild symptoms of tissue damage (Fig. 11.1).

11.3.1 Bipolaris sorokiniana

Under favourable conditions *Bipolaris sorokiniana* infects not only leaves but also roots, crowns and spikes of barley. However, for the sake of simplicity and because most interaction data were obtained in leaves, the other potentially interacting tissues will be ignored in the following. The pathogen is a typical hemibiotroph and exhibits a first biotrophic phase that is confined to single, first attacked epidermal cells before it switches to necrotrophy.

Cytological analyses of biotrophic and necrotrophic phases have been performed with special emphasis on H_2O_2 production as reflected by the oxidation of infiltrated DAB. This showed that a certain fraction of epidermal cells of susceptible barley cv. Ingrid responds to appressorial penetration attempts with DAB-stained cell-wall swellings and whole-cell oxidative burst from 24 h.a.i. onwards, and the latter reaction was reported to reduce fungal infection (Kumar et al. 2002). However, as soon as the fungus has entered the necrotrophic phase, massive H_2O_2 formation in mesophyll was observed, and this reaction was found to be positively and negatively correlated with enhanced susceptibility of *mlo5* and *bst1* mutants, respectively (Persson et al. 2009; Kumar et al. 2001). In summary, H_2O_2 formation appears to play an ambivalent role during the barley/*B. sorokiniana* interaction: It may confer resistance during the biotrophic phase and be induced or generated by the pathogen during the necrotrophic phase in order to provoke cell death. It appears more likely that the mesophyll burst is induced by the fungus because barley also responded to injected culture filtrate with H_2O_2 formation, again with a more intense reaction of *mlo5* mutant plants.

There exists one public dataset of parallel transcript profiling in resistant wild barley leaves (*H. vulgare* ssp. *spontaneum*) during attack by *B. sorokiniana* (Millett et al. 2009). Reanalysis of these data using the same statistical approach as described before (static match, >2-fold regulation, $p < 0.05$, FDR < 0.05, Zellerhoff et al. 2010) revealed a total of 1,263 up- and 666 downregulated transcripts. Similar to what was observed during the response to powdery mildew and rust attack, major functional categories of upregulated transcripts were (in decreasing order of abundance) PR response, stress, signalling, protein synthesis and degradation, as well as amino acid metabolism. Among the downregulated transcripts components of photosynthesis, RNA transcription/degradation and protein synthesis/degradation were most abundant. The similarity of this transcriptional profile to the one following challenge by the above-discussed two obligate biotrophic fungi may be unexpected but could be related to the fact that the studied interaction was resistant. We should also bear in mind the cytological similarity of the early resistance responses of epidermal cells to *B. sorokiniana* and *Bgh*, which includes the formation of local cell-wall appositions and hypersensitive cell death.

11.3.2 Pyrenophora teres

The *P. teres* pathogen comes in two flavours: The *forma teres* causes net blotch disease characterized by typical dark brown blotches that grow along the leaf axis with occasional rectangle striations causing the net-type disease pattern; the *forma maculata* on the other hand causes oval-shaped, dark brown leaf lesions. There is cytological evidence that f. *maculata* is a hemibiotrophic pathogen, whereas f. *teres* is more behaving like a necrotrophic one not always forming biotrophic primary infection vesicles and causing cellular disintegration of attacked epidermal cell early during the interaction (Liu et al. 2011).

P. teres directly penetrates the barley epidermal cell walls by appressoria, and especially f. *maculata* established biotrophic primary vesicles in first attacked cells. Subsequently secondary infection vesicles form within the same cell, which are accompanied by cell death and give rise to fast-growing infection hyphae that penetrate into the mesophyll. This morphological transformation marks the switch from biotrophic to nectotrophic fungal growth. As mentioned above, f. *teres* often does not form primary vesicles but starts its cell-destroying business as nectrotrophic pathogen right from the beginning of the interaction (Liu et al. 2011) and might thus be regarded as a conditional hemibiotrophic pathogen.

Similar to susceptible interactions with *Bgh*, barley responded to a compatible *P. teres* f. *teres* isolate with enhanced formation of ROS as reflected by NBT staining in epidermal cells. Interestingly, this was accompanied by a strongly reduced host superoxide dismutase activity that would be required to detoxify the superoxide radical (Able 2003). Therefore, also *P. teres*, at least the f. *teres*, appears to strive on ROS to invade its host. On the other hand, barley also formed cell-wall appositions in epidermal cells attacked by *P. teres* f. *maculata*, and the intensity of this response was found to be correlated with resistance induced by a primary inoculation with *Bipolaris maydis* suggesting that very early during the interaction, the same universal type of resistance response attributed to PAMP-triggered immunity also contributes to warding off *P. teres* (Jorgensen et al. 1998). It would be interesting to test if cell-wall appositions are involved in early resistance responses to *P. teres* f. *teres*, too.

Barley gene expression patterns during susceptible or resistant interactions with *P. teres* f. *teres* as well as f. *maculata* have been examined but not yet on a genome-wide scale. Thus, published information is either biased by knowledge-based selection of hybridization probes or represents a very narrow selection of strong host transcriptome changes (suppression subtractive hybridization, SSH) thereby preventing a stringent comparison with barley responses to other pathogens. Bearing the limitation of published SSH data in mind, it looks as if many of the confirmed 28 upregulated transcripts were clearly associated with the resistant response and enriched in detoxifying enzymes as well as signalling components (Bogacki et al. 2008). Fungal toxin decontamination might thus be important for this disease, which is in line with the fact that Tox A–C of the related *P. tritici-repentis* attacking wheat belong to the most prominent and best examined fungal

toxins. In wheat, a Tox A-binding protein was identified as thylakoid internal protein (Manning et al. 2007). Currently there exists one report only on the identification of the Tox A gene in *P. teres* without information about its importance in barley infection (Leisova-Svobodova et al. 2010).

11.3.3 Magnaporthe oryzae

The early infection process of *M. oryzae* in barley is quite similar to the ones of *B. sorokiniana* and *P. teres* f. *maculata*: After the germination of conidiospores on the leaf surface, darkly pigmented appressoria push penetration hyphae across the epidermal cell wall in order to establish biotrophic primary hyphae inside the first penetrated cell. Approximately 36–48 h.a.i., the fungus forms secondary invasive hyphae that grow out into neighbouring epidermal and mesophyll cells. To what extent this also represents the switch to necrotrophic growth is not clear because hallmarks of both ongoing biotrophy and necrotrophy can be found in infected barley leaves (Jarosch et al. 2005; Parker et al. 2009). According to Khang et al. (2010), infected cells stay alive until the fungus grows into the next cell. At that point the left-behind cell dies, and this might be caused by the release from effector-caused cell death suppression. Host cell death thus spreads and becoms visible as blast lesions from a certain extent of fungal tissue ramification on. The asexual life cycle is completed by approximately 4 d.a.i. when conidiospores are formed in the greyish centre of blast lesions.

The plant's responses to *M. oryzae* have been characterized in considerable detail and include cell-wall modifications, cell death, generation of ROS, transcriptional and metabolic changes and the role of selected host genes as revealed by silencing or mutation (Jarosch et al. 2005; Zellerhoff et al. 2006). Under the microscope, barley epidermal cells respond to fungal penetration attempts with the formation of cell-wall appositions, similar to many other directly penetrating pathogens. At 3–4 d.a.i. about 50 % of susceptible epidermal cells containing invasive hyphae have died as reflected by strong whole-cell autofluorescence. Also in the mesophyll, increasing numbers of cells were found to strongly autofluoresce reflecting successful fungal invasion. Epidermal barley cells exhibiting nonhost resistance to *M. grisea* strains, which infect grass species other than rice or *Triticeae* crops, were reported to respond similar to a susceptible interaction, but this was followed neither by mesophyll invasion nor cell death suggesting a block in the nonhost to grow out of primary infected epidermal cells (Zellerhoff et al. 2006). In contrast to the other hemibiotrophic and necrotrophic pathogens discussed here, barley responded to *M. oryzae* colonization with barley any formation of H_2O_2, and this was recently attributed to active ROS detoxification by a glutathione peroxidase of the fungus (Zellerhoff et al. 2008; Huang et al. 2011). These observations rather argue against an important role of *M. oryzae* toxins and support the notion that the fungus maintains a "quasi-biotrophic" interaction until late during the infection process, which might be

characterized by nutrient uptake from living cells while tolerating surrounding host cell death and maybe also recycling of nutrients leaking out from decayed host tissue. In line with the proposed "quasi-biotrophic" lifestyle of *M. oryzae* on barley, it was observed that a primary inoculation with the bacterial pathogen *Pseudomonas syringae* resulted in acquired resistance to subsequent fungal attack, which was characterized by transcriptional changes resembling salicylic acid-mediated systemic acquired resistance (SAR) against biotrophs in *Arabidopsis* as well as chemically induced resistance against powdery mildew in barley and wheat (Colebrook et al. 2012).

So far one study reported on multiparallel transcript profiling during susceptible and nonhost-resistant barley–blast interactions, respectively (Zellerhoff et al. 2010). The transcriptional response of susceptible host epidermis was rather weak (ca. 1 % of spotted cDNA clones giving rise to regulated transcriptional signals) and not comparable to the response of the same tissue to *B. graminis* infection (ca. 20 % regulated). In the nonhost interaction, the response was more intense and included ca. 150 additional regulation events. Pathway annotation by MapMan revealed that, unlike several other barley–pathogen interactions, the amino acid metabolism appeared not heavily affected at the transcript level. Instead, lipid metabolism was found to contain many regulated transcripts of corresponding enzymes and transporters such as nonspecific lipid-transfer proteins (Zellerhoff et al. 2010). In an elegant study of susceptible *M. oryzae* interactions with three different host grass species including barley, a number of important results were obtained (Parker et al. 2009). First, pools of defence-related metabolites were strongly affected by infection including alanine, aspartate, phenylalanine and malate but also of quinate indicating inhibited metabolic flow into the shikimate pathway for the accumulation of phenylpropanoids and lignin precursors. Whether this inhibition was due to fungal effector activity remains to be examined. The accumulation of amino acids during the infection was not reflected by a prominent regulation of transcripts of corresponding biosynthetic enzymes, as discussed above, and might indicate important roles of modulation of protein turnover or enzyme activities. In addition, these results clearly highlight the urgent need for "trans-omic" approaches for a more comprehensive picture of the critical events in pathogen-attacked plant cells. Primary metabolism was also affected and supported the notion that a lot of metabolic activity was channelled into export of glucose, fructose, glutamate and aspartate to fungal invasive hyphae.

In addition to more general descriptions of barley responding to *M. oryzae* or *M. grisea*, the role of some genes has been specifically addressed. The susceptibility factor *HvMlo*, which plays a very important role in the barley–*B. graminis* interaction, was also found to affect the interaction with *M. oryzae* (Jarosch et al. 1999). Interestingly, while mutated loss-of-function *mlo* alleles cause race-nonspecific and durable resistance to *B. graminis*, they result in super-susceptibility to *M. oryzae* suggesting an ambivalent role of the encoded protein depending on the fungal pathogen species. Interestingly, *HvRor1* that was identified as important downstream component of *mlo*-mediated powdery mildew resistance was also found to be required for residual resistance to *M. oryzae*, especially in the super-susceptible

mlo background. In the wild-type *Mlo* background, on the other hand, a downstream component of *Mla*-mediated race-specific resistance to powdery mildew, *HvRar1* encoding a co-chaperone for *Mla* proteins, was more important than *HvRor1* (Jarosch et al. 2005). This might indicate the involvement of partially functional NB-LRR-type *R*-genes in basal defence of wild-type barley to *M. oryzae*. Taken together, the mutant data suggest a complex interplay and convergence at some point of *HvMlo* and NB-LRR-triggered pathways in blast-attacked barley cells. The RHO-like monomeric G protein HvRAC1 was found to enhance resistance of barley to *M. oryzae* when overexpressed as constitutively active mutant version in transgenic plants (Pathuri et al. 2008). In contrast, the same protein supported accommodation of *B. graminis* haustoria again highlighting ambivalent roles of specific defence-related cellular components of one plant against different pathogens. Silencing of *HvCEBiP* encoding a putative chitin receptor in transgenic barley resulted in hypersusceptibility to *M. oryzae* thus strongly suggesting a role of recognition of this PAMP for basal defence (Tanaka et al. 2010).

11.4 Response to Necrotrophic Pathogens

The necrotrophic and hemibiotrophic lifestyles of some fungi are sometimes not easily separable because both kill invaded host tissue at final stages of infection. However, if morphologically distinct biotrophic primary hyphae are absent and if toxins are produced essentially from the initial stages of infection onwards, we can infer that the pathogen is necrotrophic (Horbach et al. 2011). It appears that true necrotrophic pathogens interact with their host(s) in a rather nonspecific manner in the sense of a "loose relationship" and are interested to rapidly and efficiently decompose their host substrate in order to maximize nutrient uptake. An interesting question to be asked is whether barley or any other plant being attacked by a necrotrophic fungus will respond to such a massive and destructive attack by more dramatic or less pronounced changes in host parameters such as altered transcript abundance. It appears possible that at least directly colonized tissue is simply left no time to mount a complex defence response because of inhibitory and apoptosis-inducing activity of rapidly released fungal toxins or ROS.

An emerging necrotic leaf-spotting disease in barley, especially in Northern Europe, is caused by *Ramularia collo-cygni* that is best classified into the group of necrotrophic pathogens based on (still limited) phytopathological data. Due to virtually still lacking information about plant responses to this pathogen, I will only briefly mention it referring to one review and some original literature addressing the pathogen and disease (Walters et al. 2008). The fungus enters its host via stomatal openings and grows in the leaf mesophyll where it induces necrosis that becomes visible as dark spots approximately 7 days after inoculation in controlled conditions (Stabentheiner et al. 2009). In the field long asymptomatic phases of infection have also been reported. The asexual infection cycle is fulfilled after release of conidia from conidiophores emerging through stomatal pores.

R. Collo-cygni is known to produce host-nonspecific, photoactive toxins called Rubellins (Miethbauer et al. 2003). Recently, *Agrobacterium*-mediated transformation of the fungus was reported, which should allow to study the growth of fluorescent protein-tagged strains inside plant tissue and to functionally address fungal gene expression in the future (Thirugnanasambandam et al. 2011).

11.4.1 Fusarium graminearum *and* F. culmorum

F. graminearum and *F. culmorum* infect wheat and barley with the prime site of infection being spikes at flowering stage. Severe infection causes considerable yield loss in both crops, which is characterized by typical shrunken "tombstone" wheat kernels or pinkish discoloration of hulled barley grains (Choo et al. 2004; Goswami and Kistler 2004; Wagacha and Muthomi 2007). In feed barley, the major grain quality problem arising from *Fusarium* head blight is related to livestock poisoning by fungus-derived trichothecene toxins, especially by deoxynivalenol (DON) or HC-2 toxin causing alimentary toxic aleukia and multiple disorders such as reproductive dysfunction at high doses and lower doses, respectively (Placinta et al. 1999; Pestka 2007). In malting barley *Fusarium*-infected grain causes quality changes and excessive foam formation (gushing) of beer, besides DON contamination that can contribute significantly to the tolerable daily DON intake of regular beer drinkers (Schwarz et al. 1996; Papadopoulou-Bouraoui et al. 2004; Oliveira et al. 2012).

F. graminearum has recently been questioned as a typically nectrotrophic pathogen in wheat due to microscopic observation of intercellularly growing, irregularly shaped hyphae at the initial stage of rachis infection that were not surrounded by dead host cells (Kazan et al. 2012; Brown et al. 2010). However, no clearly distinct phases of initial wheat infection could be discriminated. Also, *F. graminearum* as well as *F. culmorum* were reported to express the genes encoding enzymes for DON toxin production from the first analysed time point (24 h.a.i.) onwards (Hallen-Adams et al. 2011; Beccari et al. 2011) and—in the case of *F. culmorum*—to kill infected wheat root tissue rapidly after inoculation. I therefore refer to both pathogen species as predominantly necrotrophic ones. *F. graminearum* first infects floral bracts and then inner parts of barley florets. In contrast to wheat it cannot spread via the rachis from infected to noninfected parts of the spike suggesting that barley as a species possesses so-called type II resistance against spreading. After a more or less extended phase of epiphytic growth, the fungus penetrates into the host tissue by inconspicuous appressorial-like structures followed by massive hyphal growth and toxin production. At approximately 48 h.a.i., brown discoloration of the lemma becomes visible as first macroscopic symptom of infection, and from 96 h.a.i. onwards inner parts of florets are heavily colonized by hyphae (Skadsen and Hohn 2004; Boddu et al. 2006). In a proteomics and transcriptomics approach focussing on the secretome of *F. graminearum* growing on wheat- or barley flour media and infected spikes, respectively, a number of cell

wall-, starch- and protein-degrading enzymes were identified substantiating the necrotrophic lifestyle of the pathogen (Yang et al. 2012).

There exists little information about clearly defined physiological or cytological responses of barley spike tissues to *Fusarium* sp. attack. Recent report suggests auxin to play an important role in defence of barley spike tissues against *F. culmorum* because hormone levels were positively correlated with resistance priming against the pathogen, besides partial protection by exogenous auxin application (Petti et al. 2012). Following a metabolomics approach, Kumaraswamy et al. (2012) identified jasmonate as being induced by a DON-producing *F. graminearum* strain but not by a *tri5* mutant defective in DON production, which was correlated with absence or lower accumulation of precursors of lignin-like cell-wall materials in spikes infected by the DON-producing strain. This opened the question about a possible disease-supporting role of the jasmonate pathway. More data are available on the host transcriptome in response to fungal inoculation as well as trichothecene treatment (Boddu et al. 2006, 2007). Fungal infection of the susceptible barley cv. Morex resulted in little transcriptional changes up to 48 h.a.i. From 48 h.a.i. onwards, DON became clearly detectable suggesting, in agreement with results from DON treatment of barley, that DON is a major factor determining host gene induction. Gene function of DON-induced transcripts could be grouped into defence-related (transport and detoxification) and in toxicity-related (protein ubiquitination and cell death control). Astonishingly, almost no downregulated transcripts were identified, in contrast to all the other barley–pathogen interactions for which transcriptome data are available. In *F. graminearum*-attacked spikes, prominent functional groups of nine upregulated UDP-glucanosyltransferase and 12 ABC transporter transcripts suggested a role of the encoded proteins in the detoxification of DON and other toxins by clycosylation and transport into vacuoles, respectively. Furthermore, many enzymes of the tryptophan/tryptamine biosynthetic pathway were transcriptionally induced, besides a range of PR proteins that are common to almost all plant–pathogen interactions. Although these studies provided valuable hints as to important aspects of the response of susceptible barley to the *Fusarium* head blight fungus, they provided no information as to the molecular basis of the widespread type II resistance in this crop (Jansen et al. 2005). The impact of DON on the host transcriptional response in the presence of the fungus was directly addressed by comparing a wild-type versus a *tri5*-mutant strain interrupted in trichothecene biosynthetic pathway and, as a consequence, less virulent on wheat and barley. This revealed more upregulated transcripts in plants attacked by the wild-type strain indicating that the toxin effect was not related to an overall weakening of host responses but triggered protein ubiquitination and cell death pathways for disease establishment on the one hand and detoxification pathways as countermeasure of the plant on the other (Boddu et al. 2007).

Proteome and metabolome studies in *F. graminearum*-attacked barley have been performed and add valuable information as to the infection strategy and possible resistance mechanisms. Yang et al. (2010) found that proteome changes in the susceptible barley cv. Scarlett were associated with the accumulation of proteolytic

degradation products of alpha-amylase and other host proteins indicating massive tissue degradation from 3 days post inoculation onwards, the time point where fungal biomass was strongly accumulating. An interesting study compared metabolite profiles during a susceptible and five quantitatively resistant interactions of one fungal isolate with different barley genotypes (Bollina et al. 2011). This lead to the identification of 16 statistically significant, resistance-related compounds including phenylpropanoid- and flavonoid pathway intermediates and derivates such as sinapic acid, naringenin and kaempferol derivates. A good part of these metabolites, which were either present in higher amounts in resistant genotypes or accumulated to higher levels upon *F. graminearum* attack, have been previously shown to exert antifungal activity in vitro. This will allow mapping corresponding pathway enzymes and searching for gene-QTL co-localization in order to focus on promising gene candidates for quantitative *Fusarium* head blight resistance in barley.

11.4.2 Rhynchosporium commune

Scald caused by *R. commune* is a serious disease especially in Northern Europe (Avrova and Knogge 2012). *R. commune* penetrates barley leaf cuticle and grows between the cuticle and the epidermal cell layer. It does not penetrate epidermal cell walls but has been shown to rapidly release toxic NIP peptides, two of which activate a barley plasmalemma H+ ATPase leading to epidermal cell collapse within 4 d.a.i. (Wevelsiep et al. 1991, 1993). This is preceded by very rapid (3 h. a.i.) $HO_2^{\cdot}/O_2^{\cdot -}$ and H_2O_2 accumulation in epidermal cells along the anticlinal walls of which runner hyphae from germinating ascospores have started to grow (Linsell et al. 2011). Because exogenously applied scavengers of ROS did not affect resistance responses but limited the growth of *R. commune*, the early whole-cell oxidative burst is most likely related to fungal pathogenicity and not to plant defence (Able 2003). Late during the infection process, collapsed epidermal cells are largely replaced by a mesh of fungal hyphae, and it is not before this stage that the mesophyll starts to collapse leading to the macroscopically visible lesions (Steiner-Lange et al. 2003).

Because of the subcuticular mode of fungal development, we may assume the epidermis to be the prime tissue for studying host responses. As already mentioned, an $HO_2^{\cdot}/O_2^{\cdot -}$ and H_2O_2 burst was observed in susceptible interactions and appeared not to contribute to plant defence. This is in agreement with infection strategies of other typical necrotrophs such as *Botrytis cinerea* or *Sclerotinia sclerotiorum* that also produce or induce H_2O_2 in order to kill cells and cause rapid leakage of nutrients. Barley possesses a number of major R-genes against *R. commune*. The NIP1 toxic peptide has been found to be recognized as AvrRrs1 by the Rrs1 resistance protein in cultivar Atlas46, which resulted in a more pronounced accumulation of some transcripts encoding PR proteins (Hahn et al. 1993; Rohe et al. 1995).

Transcripts of some PR protein genes analysed on northern blots accumulated either in leaf epidermis or mesophyll within 72 h.a.i. suggesting that some infection- or defence-related signals also reach the inner leaf before epidermal collapse (Steiner-Lange et al. 2003). A parallel transcript-profiling approach in peeled leaf epidermis using a 10K spotted cDNA array and robust rank product analysis (FDR < 0.05) revealed 94 upregulated and 104 downregulated transcripts during the first 48 h.a.i. in the susceptible cultivar Atlas or the resistant near-isogenic line Atlas46 carrying *Rrs1* attacked by an *AvrRrs1*-carrying *R. commune* isolate. (J. Basak, W. Knogge and P. Schweizer, unpublished). Among the most robustly upregulated transcripts, we found typical PR proteins such as germin-like family members, class III peroxidases, chitinase, glutathione S-transferase and GRP94 that had been reported to be induced and relevant for the barley *Bgh* interaction (Thordal-Christensen et al. 2000). Most strongly downregulated transcript encode several components of primary metabolism such as glycolytic enzymes and photosynthetic proteins including RuBisCO and ribosomal proteins, besides many unknown proteins. The relatively weak transcriptional response of barley epidermis (2 % regulated transcripts) is in sharp contrast to observations during the interaction with *Bgh* or *Bgt* (ca. 20 % regulated transcripts) but resembles results obtained during the first 48 h after attack of susceptible barley spikes with the necrotrophic pathogen *F. graminearum* (ca. 0.7 % regulated transcripts). It may thus be speculated that this behaviour is related to the necrotrophic lifestyle of both *R. commune* and *F. graminearum*, which either evade strong host recognition due to "loose" interactions or by efficiently suppressing PAMP-triggered defences with the help of toxins released from the very beginning of the interaction. A comparison of wild-type and mutant strains differing in toxin production may help to answer this question, similar to the discussion in the context of the barley/*F. graminearum* interaction.

11.5 Comparison of Interactions

We have seen that barley responds differentially to attack by different fungal pathogens. Ideally, in order to compare these responses and relate them to the respective pathogen species and its infection strategy, direct comparative, multifactorial studies are designed using different fungi and one or a common set of barley genotypes. Such studies have been rarely carried out until now thus limiting the stringency one may apply to the comparison of the measured infection or response parameters. However, some meta-information across barley–pathogen interactions may be useful as summarized below.

At the cellular level, the formation of cell-wall appositions at sites of attempted fungal penetration is one of the most widespread and rapid responses and was described to occur in all the obligate biotrophic and hemibiotrophic interactions. The absence from interactions with the two necrotrophic pathogens *F. graminearum*/*F. culmorum* and *R. commune* might be related to the absence of

a clearly defined cell-wall penetration by these two fungi. The formation of cell-wall appositions is assumed to represent one of the microscopically visible outcomes of PAMP-triggered immunity, which would explain its widespread occurrence. The same is likely true for the general transcriptional host response, as discussed above. The formation of ROS is another widespread response of barley to attack by most fungal pathogens. Here, however, one has to differentiate between PAMP-triggered formation in terms of (non)host defence responses and pathogen-derived or pathogen-triggered formation, which probably reflects toxin action during necrotrophic interactions. Extreme examples of detrimental *versus* beneficial functions of ROS for fungal virulence are the rapid (within 3–6 h.a.i.) H_2O_2 production by epidermal cells attacked by *R. commune* and *B. graminis*, respectively (Huckelhoven et al. 1999; Linsell et al. 2011). Less clear is the picture with respect to superoxide radical because virulent isolates both of the biotrophic pathogen *Bgh* as well as the predominantly necrotrophic *P. teres* f. *teres* were found to increase the accumulation of this ROS (Huckelhoven and Kogel 1998; Able 2003). If both virulent pathogens indeed actively trigger a superoxide radical burst, then *Bgh* might aim at suppressing defence responses by inducing host-encoded ROS scavengers, whereas *P. teres* might intend to induce host cell death by producing or triggering toxic doses of this ROS. In all these described scenarios, a good deal of genomic and gene-oriented research will still be required before a clearer picture of the major regulators of ROS production and their modes of action can emerge. One electrophysiological study compared the effect of *Bgh* versus *B. sorokiniana* infection on barley cell membrane potentials and apoplastic leaf pH (Felle et al. 2008). Interestingly, the substantial increase in leaf apoplast pH during a susceptible interaction with *B. sorokiniana* more resembled a resistant interaction with *Bgh* mediated by the *Mla* resistance gene as compared to the corresponding susceptible *Bgh* interaction that left pH more or less unaffected. This lends further support to the model of apoptotic pathway triggering by virulent hemibiotrophic or necrotrophic pathogens such as *B. sorokiniana*, which otherwise serve to stop avirulent biotrophic pathogens such as the *Bgh*. Along these lines there are few interesting examples of opposite mutant or transgene effects depending on the interacting fungal pathogen. First, *mlo*-mutant barley was reported to be highly resistant to Bgh but at the same time super-susceptible to the hemibiotrophic fungi *M. oryzae* as well as *B. sorokiniana* (Kumar et al. 2001). A similar ambivalence of (trans)gene function was found by comparing basal resistance of transgenic plants overexpressing either *Bax inhibitor-1* or a constitutively active form of the small G-protein *RACB* after inoculation with *Bgh*, *M. oryzae* or *F. graminearum* (Pathuri et al. 2008; Babaeizad et al. 2009). These examples were discussed with respect to opposing gene function requirements for accommodating obligate biotrophic or hemibiotrophic fungal pathogens: The cell death-suppressing proteins Mlo and Bax inhibitor-1 support the accommodation of *Bgh* haustoria but counteract cell death responses triggered by *Fusarium* sp. and *M. oryzae* later during successful tissue colonization.

Parallel (ideally genome-wide) transcript profiling is an efficient approach to find commonalities and differences of a single plant species interacting with different pathogens. Figure 11.2 represents a preliminary meta-analysis of public

a

		% Of all unigenes in assigned superbins														
		Obligate biotrophic								Hemibiotrophic			Necrotrophic			
Nr.	Superbin	Bgh_EPI_I	Bgt_EPI_I	Bgh_L_m	Bgh_L_M	Ph_I	Ph_L94	Pt_I	Ph_Vada	Mory_I	Mgris_I	Bipol_Hsp	FHB_Mor	R.comm	PGRC1	Barley1
1	PS	0,0	0,5	0,1	0,3	0,6	0,3	0,8	0,0	0,0	0,0	0,3	0,7	0,0	2,0	2,6
2	Major CHO metabolism	1,7	1,5	0,9	0,9	1,8	0,6	2,3	0,6	4,5	1,7	0,6	0,4	5,3	1,3	1,1
3	Minor CHO metabolism	0,8	0,5	0,1	0,2	1,8	0,6	2,3	0,3	0,0	0,0	0,3	0,0	5,3	1,1	1,1
4	Glycolysis	0,8	0,5	0,9	0,3	0,6	0,3	0,8	0,0	0,0	0,0	1,6	0,4	0,0	0,9	0,8
5	Fermentation	0,8	0,5	0,0	0,0	0,6	0,0	0,8	0,3	0,0	1,7	0,6	0,4	0,0	0,3	0,2
6	Gluconeogenese/ glyoxylate cycle	0,0	0,5	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,4	0,0	0,1	0,1
7	OPP	1,7	1,0	0,8	0,8	2,4	0,0	2,3	0,3	0,0	0,0	1,0	0,0	2,6	0,4	0,4
8	TCA / org. transformation	0,8	1,0	0,7	0,8	1,2	0,3	0,0	0,3	0,0	1,7	0,6	0,7	0,0	0,9	0,7
9	Mitoch.e- transport / ATP synthesis	0,0	1,5	1,1	1,2	0,6	0,9	0,8	1,0	0,0	0,0	0,9	1,1	0,0	1,3	1,2
11	Lipid metabolism	1,7	2,0	3,7	3,8	1,8	1,5	3,9	1,0	9,1	11,9	3,9	1,8	5,3	2,9	3,2
12	N-Metabolism	0,0	0,5	0,0	0,0	0,0	0,3	0,0	0,0	0,0	0,0	0,4	0,0	0,0	0,2	0,2
13	Amino acid metabolism	8,3	6,1	4,6	4,9	12,4	2,9	13,2	4,2	0,0	3,4	7,4	7,0	2,6	3,0	3,4
14	S-Assimilation	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,1	0,0	0,1	0,1
15	Metal handling	0,0	1,0	0,2	0,2	0,0	0,6	0,0	0,3	0,0	0,0	1,0	1,1	0,0	0,4	0,6
16	Secondary metabolism	6,7	6,1	5,9	5,9	6,5	7,6	4,7	5,5	0,0	3,4	5,3	8,5	5,3	2,4	3,1
18	Co-factor and vitamine metabolism	0,8	0,5	0,3	0,4	0,6	0,3	0,0	0,0	0,0	0,0	0,3	0,4	0,0	0,3	0,3
19	Tetrapyrrole synthesis	0,0	0,0	0,1	0,1	0,0	0,3	0,0	0,0	0,0	0,0	0,4	0,4	0,0	0,5	0,4
22	Polyamine metabolism	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,2	0,2
23	Nucleotide metabolism	2,5	2,0	1,5	1,6	1,8	0,9	2,3	0,6	0,0	0,0	1,1	0,0	0,0	1,4	1,4
24	Biodegradation of Xenobiotics	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,2	0,2
25	C1-Metabolism	0,0	0,0	0,5	0,4	0,6	0,0	0,0	0,0	0,0	0,0	0,4	0,0	0,0	0,3	0,3
27	RNA	2,5	5,1	7,3	8,1	1,8	8,7	2,3	8,4	4,5	5,1	4,0	8,5	10,5	14,5	13,7
28	DNA	0,0	0,5	1,1	1,6	0,0	0,9	0,0	1,6	0,0	1,7	0,9	1,1	0,0	3,3	4,1
29	Protein	11,7	18,2	16,0	15,4	8,8	6,1	7,8	6,2	9,1	8,5	11,5	3,3	15,8	23,8	21,9
31	Cell	2,5	2,5	4,6	3,9	3,5	0,6	3,1	0,6	0,0	1,7	1,6	0,7	2,6	4,4	3,8
33	Development	0,0	1,5	0,9	0,9	0,0	1,7	0,0	1,3	0,0	1,7	1,1	1,8	0,0	2,8	3,0
17	Hormone metabolism	3,3	2,5	3,6	3,6	2,9	5,0	3,1	5,5	13,6	3,4	3,9	4,8	0,0	2,8	3,0
34	Transport	6,7	5,1	7,2	7,7	7,6	9,3	7,8	7,8	13,6	10,2	7,0	8,1	5,3	4,9	5,3
10	Cell wall	2,5	1,5	2,5	2,2	1,8	1,7	2,3	2,3	0,0	0,0	2,0	1,1	0,0	2,5	2,2
30	Signalling	5,8	7,1	13,1	12,8	9,4	15,7	10,9	13,0	0,0	5,1	11,5	5,9	5,3	6,7	6,0
21	Redox.ascorbate and glutathione	6,7	4,5	2,0	2,2	5,3	1,5	4,7	1,9	4,5	6,8	2,3	1,1	0,0	1,7	1,5
20	Stress	12,5	9,6	8,5	8,1	9,4	14,3	8,5	14,6	18,2	15,3	11,5	14,0	15,8	4,2	5,0
26	Misc incl. PR	19,2	16,2	11,6	11,7	16,5	17,2	15,5	22,1	22,7	16,9	16,8	25,1	18,4	8,3	8,7
	Number of assigned unigenes	**120**	**198**	**887**	**899**	**170**	**343**	**129**	**308**	**22**	**59**	**799**	**271**	**38**	**3967**	**12014**

Fig. 11.2 (continued)

b

		% Of all unigenes in assigned superbins														
		Obligate biotrophic								Hemibiotrophic			Necrotrophic			
Nr.	Superbin	Bgh_EPI_I	Bgt_EPI_I	Bgh_L_m	Bgh_L_M	Ph_I	Ph_L94	Pt_I	Ph_Vada	Mory_I	Mgris_I	Bipol_Hsp	FHB_Mor	R.comm	PGRC1	Barley1
1	PS	5,7	9,3	11,9	10,2	16,6	3,7	21,1	0,0	20,0	25,9	16,2	n.a.	42,6	2,0	2,6
2	Major CHO metabolism	0,0	2,3	1,2	1,8	0,7	2,4	2,1	0,0	0,0	1,9	0,8	n.a.	0,0	1,3	1,1
3	Minor CHO metabolism	2,9	2,3	2,3	2,2	3,3	3,7	1,1	0,0	0,0	1,9	1,3	n.a.	0,0	1,1	1,1
4	Glycolysis	2,9	0,6	0,5	0,8	1,3	0,0	0,5	0,0	0,0	0,0	0,5	n.a.	2,1	0,9	0,8
5	Fermentation	0,0	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	n.a.	0,0	0,3	0,2
6	Gluconeogenese/ glyoxylate cycle	0,0	0,6	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	n.a.	0,0	0,1	0,1
7	OPP	2,9	1,7	0,3	0,2	0,0	0,0	0,0	0,0	0,0	0,0	0,3	n.a.	0,0	0,4	0,4
8	TCA / org. transformation	2,9	0,6	0,3	0,3	0,0	0,0	0,5	0,0	0,0	1,9	0,5	n.a.	2,1	0,9	0,7
9	Mitoch.e- transport / ATP synthesis	0,0	1,7	0,1	0,2	0,7	0,0	1,1	0,0	0,0	0,0	0,0	n.a.	0,0	1,3	1,2
11	Lipid metabolism	14,3	2,9	3,9	5,3	4,0	2,4	4,2	5,3	13,3	1,9	5,3	n.a.	2,1	2,9	3,2
12	N-Metabolism	2,9	0,6	0,2	0,1	0,7	0,0	0,0	0,0	0,0	1,9	0,3	n.a.	0,0	0,2	0,2
13	Amino acid metabolism	0,0	4,7	2,3	1,9	2,6	1,2	1,1	0,0	0,0	3,7	2,3	n.a.	2,1	3,0	3,4
14	S-Assimilation	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	n.a.	0,0	0,1	0,1
15	Metal handling	2,9	0,6	0,5	0,7	0,0	0,0	0,5	0,0	0,0	0,0	0,5	n.a.	0,0	0,4	0,6
16	Secondary metabolism	0,0	2,3	3,3	3,8	2,0	3,7	2,1	10,5	13,3	7,4	5,6	n.a.	2,1	2,4	3,1
18	Co-factor and vitamine metabolism	0,0	0,6	0,8	0,4	0,7	0,0	0,5	0,0	0,0	0,0	0,5	n.a.	0,0	0,3	0,3
19	Tetrapyrrole synthesis	0,0	0,0	1,2	1,4	0,7	1,2	1,1	0,0	0,0	0,0	3,0	n.a.	0,0	0,5	0,4
22	Polyamine metabolism	0,0	0,6	0,0	0,0	0,0	2,4	0,0	5,3	0,0	0,0	0,3	n.a.	0,0	0,2	0,2
23	Nucleotide metabolism	0,0	1,2	1,3	1,0	1,3	0,0	1,1	0,0	0,0	1,9	0,3	n.a.	0,0	1,4	1,4
24	Biodegradation of Xenobiotics	0,0	0,0	0,2	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	n.a.	0,0	0,2	0,2
25	C1-Metabolism	0,0	0,0	0,4	0,4	0,0	0,0	1,1	0,0	0,0	0,0	0,3	n.a.	2,1	0,3	0,3
27	RNA	11,4	13,4	13,6	14,8	13,2	12,2	11,6	21,1	0,0	5,6	11,2	n.a.	8,5	14,5	13,7
28	DNA	5,7	1,7	2,0	2,9	2,6	6,1	2,1	5,3	0,0	1,9	1,3	n.a.	2,1	3,3	4,1
29	Protein	11,4	24,4	17,7	17,4	17,9	13,4	18,4	15,8	13,3	9,3	11,9	n.a.	17,0	23,8	21,9
31	Cell	0,0	2,3	3,8	3,3	2,6	0,0	3,2	0,0	0,0	0,0	2,5	n.a.	0,0	4,4	3,8
33	Development	5,7	2,3	2,2	2,4	3,3	3,7	2,6	5,3	0,0	0,0	2,5	n.a.	0,0	2,8	3,0
17	Hormone metabolism	2,9	1,2	2,0	2,2	2,6	8,5	2,6	15,8	6,7	1,9	5,1	n.a.	0,0	2,8	3,0
34	Transport	8,6	6,4	5,1	4,5	4,6	1,2	4,2	0,0	13,3	5,6	4,1	n.a.	2,1	4,9	5,3
10	Cell wall	2,9	2,9	2,6	3,8	3,3	7,3	3,2	0,0	0,0	3,7	5,8	n.a.	6,4	2,5	2,2
30	Signalling	2,9	2,3	3,6	4,4	5,3	3,7	5,3	0,0	6,7	1,9	3,0	n.a.	4,3	6,7	6,0
21	Redox.ascorbate and glutathione	0,0	1,7	2,4	1,9	0,7	1,2	2,1	0,0	0,0	1,9	1,0	n.a.	0,0	1,7	1,5
20	Stress	5,7	2,3	4,2	3,4	4,6	13,4	3,2	5,3	13,3	11,1	6,1	n.a.	2,1	4,2	5,0
26	Misc incl. PR	5,7	6,4	9,7	7,9	4,6	8,5	3,7	10,5	0,0	9,3	7,6	n.a.	2,1	8,3	8,7
	Number of assigned unigenes	**35**	**172**	**915**	**906**	**151**	**82**	**190**	**19**	**15**	**54**	**394**	**n.a.**	**47**	**3967**	**12014**

Fig. 11.2 (continued)

Fig. 11.2 (continued) Over- or underrepresentation of pathogen-regulated barley transcripts belonging to different functional categories. (**a**) The *colour-coded relative number* of upregulated transcripts belonging to different functional categories is shown following inoculation with fungal pathogens. For functional categorization the MapMan binning system was used. The relative number of transcripts present on two transcript-profiling arrays (barleyPGRC1 from IPK and Barley1 from Affymetrix) are shown at the *right-hand side* of the panel. Please note the high similarity between the two arrays with respect to the distribution of spotted features among the functional categories. Bgh_EPI_I, peeled epidermis from *B. graminis* f.sp. *hordei*-attacked cv. Ingrid (susceptible); Bgt_EPI_I, peeled epidermis from *B. graminis* f.sp. *tritici*-attacked cv. Ingrid (resistant); Bgh_L_m, leaves from *B. graminis* f.sp. *hordei*-attacked *mla* mutants (susceptible); Bgh_L_M, leaves from *B. graminis* f.sp. *hordei*-attacked wild-type plants each containing one of three different *Mla* alleles (resistant); Ph_I, leaves from *Puccinia hordei*-attacked cv. Ingrid (susceptible); Ph_L94, leaves from *Puccinia hordei*-attacked landrace. L94 (susceptible); Pt_I, leaves from *Puccinia triticina*-attacked cv. Ingrid (resistant); Ph_Vada, leaves from *Puccinia hordei*-attacked cv. Vada (partially resistant); Mory_I, peeled epidermis from *Magnaporthe oryzae*-attacked cv. Ingrid (susceptible); Mgris_I, peeled epidermis from *Magnaporthe grisea*-attacked cv. Ingrid (resistant); Bipol_Hsp, leaves from *Bipolaris sorokiniana*-attacked wild barley (*H. spontaneum*, resistant); FHB_Mor, spikelets from *Fusarium graminearum*-attacked cv. Morex (susceptible); R.comm., leaf epidermis from *Rhynchosporium commune*-attacked cv. Atlas (susceptible) and Atlas46 (resistant). Experiments Bgh_EPI_I, Bgt_EPI_I, Ph_I, Pt_I, Mory_I and Mgris_I are described in Zellerhoff et al. (2010); Bgh_L_m and Bgh_L_M in Moscou et al. (2011a); Ph_L94 and Ph_Vada in Chen et al. (2010b); Bipol_Hsp in Millett et al. (2009); FHB_Mor in Boddu et al. (2006); R.comm., refers to unpublished data by Basak, Knogge and Schweizer. *CHO* carbohydrates, *Misc* miscellaneous, *OPP* oxidative pentose phosphate metabolism, *PR* pathogenesis related, *PS* photosynthesis. TCA tricarboxylic acid cycle. (**b**) The same experimental setup and publications as described in (**a**) but showing downregulated transcripts

plus own unpublished datasets of barley–pathogen interactions and was achieved by linking sets of regulated genes with functional bin information derived from the barley MapMan tool. In order to estimate if regulated transcripts might be over-or under represented in a given superbin, they have to be compared to the overall transcript abundance in that superbin across the entire arrays used for these studies (last two columns in Fig. 11.2). Among the upregulated transcripts, those belonging to superbins "amino acid metabolism", "secondary metabolism", "signalling", "stress" and "miscellaneous incl. PR" appeared to be over-represented in a majority of studied interactions, irrespective of fungal species or lifestyle. Conversely, transcripts belonging to "photosynthesis" and "RNA" were under-represented. This demonstrates a remarkably robust general stress and defence response, which is most likely triggered by a bouquet of highly conserved fungal PAMPs. However, there were exceptions: Transcript abundance in "lipid metabolism" was clearly over-represented in both interactions of barley with *M. oryzae* and *M. grisea*, whereas almost no regulated genes were found in "nucleotide metabolism" during interactions with hemibiotrophic or necrotrophic fungi. Because samples of barley interacting with obligate biotrophic fungi were derived both from peeled epidermis and from entire leaves, tissue type cannot be the explanation to this difference.

Among the downregulated transcripts there was a strong over-representation of those belonging to "photosynthesis", again independent of fungal lifestyle. This suggests that attacked barley inevitably switches away from carbon assimilation to (catabolic) stress metabolism, as suggested earlier. No clearly visible trend across several pathosystems could be found for the other superbins. One interesting tissue-specific pattern was a strong over-representation of "lipid metabolism" in epidermis attacked by compatible powdery mildew or blast fungi. This might be related to the fact that cuticle and waxy layers are delivered by epidermal cells and suggests that downregulation of their biosynthetic pathway is somehow involved in disease establishment. During its interactions with hemibiotrophic fungi, barley appears to have downregulated an enhanced proportion of transcripts belonging to "secondary metabolism". However, it appears currently not possible to rationalize such behaviour.

In summary, most transcriptional responses of barley look similar across all tested pathosystems, at least at the level of signalling, metabolic or catabolic pathways. Most likely, when breaking the comparison down to individual transcripts, overlap will decrease significantly as shown recently in a direct comparison of six barley–pathogen interactions (Zellerhoff et al. 2010). There were also a number of interesting exceptions to this general response schema at pathway level, and these deserve further attention in the future because they might provide clues to specific adaptations of different pathogens allowing them to invade barley. Alternatively they could offer clues of specific plant defence responses to ward off the different intruders.

11.6 Resistance Mechanisms

Many of the discussed reactions of barley to different fungi reflect more or less successful defence of the plant against pathogenic attack. To what extent there exists a true specificity of responses to different pathogens or whether observed differences, e.g. in transcriptional profiles, rather reflect the difference between a nonspecific, general host response minus pathogen-specific patterns of effector-mediated defence suppression remains to be vigorously tested. It appears clear, however, that barley lines up with many other plant species in terms of the current co-evolutionary model of plant innate immunity (Jones and Dangl 2006). According to the model, PAMP-triggered immunity (PTI) is the basis for strong and durable resistance against most non-adapted pathogens that have not co-evolved with a specific plant species such as barley. A few co-evolving host pathogens managed to suppress the critical components of PTI by secreted effector molecules thus establishing what is also known as "basic compatibility". Effector-mediated defence suppression is not complete and varies depending on the allelic status of host genes underlying the many resistance QTL that have been identified until present. Because QTL-mediated resistance was found to act against many pathogen races, I will refer to it as race-nonspecific host resistance (NR) throughout. In barley causative genes for resistance QTL have so far not been molecularly isolated. Superimposed on the genetically complex NR, plants evolved a highly specific recognition system for effector molecules as well as effector–target complexes that is based on nucleotide-binding leucine-rich repeat (NB-LRR) proteins and referred as "effector-triggered immunity" (ETI). Unlike NR ETI usually provides complete protection, but due to differences in functionally redundant effector bouquets of pathogen races, it was found to be race-specific and nondurable. This nondurability appears to be a logical consequence of the extreme selection pressure exerted by high levels of host resistance combined with the ease of escaping from the stress by mutating or deleting non-essential effector-encoding genes on the pathogen side.

11.6.1 Nonhost Resistance

Nonhost interactions of barley to non-adapted pathogens such as powdery mildew, rust and blast fungi infecting other grass species have been described at the genetic, transcriptional and cellular levels and have also been functionally examined with respect to the role of few selected genes (Neu et al. 2003; Eichmann et al. 2004; Trujillo et al. 2004; Jafary et al. 2006, 2008; Zellerhoff et al. 2006; Aghnoum and Niks 2010).

A major advance for a better understanding of the genetic basis of nonhost resistance was achieved in the barley–rust interaction where genes for nonhost susceptibility were accumulated by repeated trans-segregant crosses resulting in

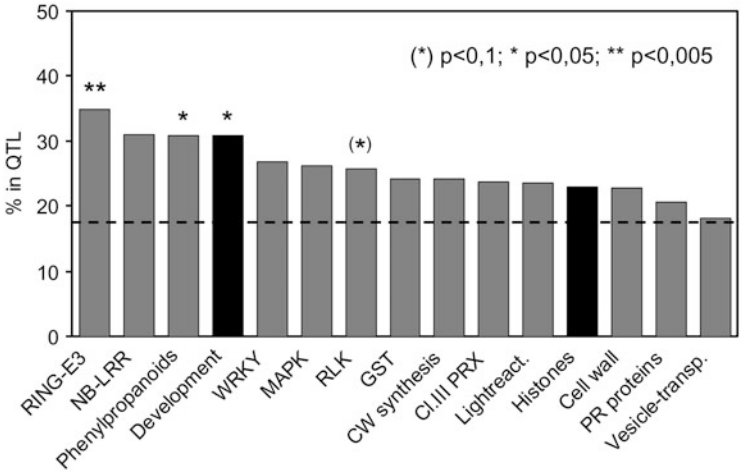

Fig. 11.3 Co-localization of functional groups of barley genes with QTL for nonhost resistance to non- or poorly adapted rust species. Functional gene groups were formed based on the MapMan binning system and tested for over-representation inside resistance QTL by Chi-square analysis. The *dotted line* represents the value of the null hypothesis. *Black columns* indicate functional bins that were selected as negative control in *Blumeria graminis*-attacked leaves because they were not expected to be strongly involved in plant defence (Schweizer and Stein 2011). CW cell wall *PRX* peroxidase, *transp*. transport, *RLK* receptor-like protein kinase, *WRKY* WRKY transcription factor, *RING-E3* "Really Interesting New Gene"-type E3 ubiquitin ligase, *PR* pathogenesis-related, *MAPK* mitogen-associated protein kinase, *GST* glutathione S-transferase; *NB-LRR* nucleotide binding-leucine rich repeat

two barley lines with high susceptibility to inappropriate rust fungi (Atienza et al. 2004). Segregation analysis of progeny resulting from a series of crosses between "normal" nonhost-resistant parents and one of the new, nonhost susceptible lines yielded first data as to the genetic basis of the (non)host status in barley. Interestingly, nonhost resistance to several rust species was inherited in a quantitative manner with a number of identified QTL, but the map position of these varied greatly depending (1) on the nonhost rust species and (2) on the genotype of the (more) nonhost-resistant parent (Jafary et al. 2006). I tested a number of MapMan functional (super)bins for significant co-localization with nonhost-resistant QTL, as recently described for host resistance QTL to the adapted Bgh. This revealed RING-E3 ubiquitin ligases, genes of the phenylpropanoid pathway, receptor-like kinases and genes of plant development as being significantly enriched within confidence intervals of nonhost resistance QTL (Fig. 11.3). The QTL co-localizing receptor-like kinases and phenylpropanoid pathway genes were previously identified as important components of PTI in several plant species suggesting this pathway to be involved in nonhost resistance, as proposed in a developed model by Schulze-Lefert and Panstruga (2011). The accumulation of susceptibility alleles in barley to *Bgt* by successive crosses was also successful and

may offer fascinating insight into the genetic basis of nonhost resistance against powdery mildew fungi (Aghnoum and Niks 2010).

Microscopic examination of nonhost resistance in barley showed that non-adapted powdery mildew fungi are usually stopped by cell-wall appositions during penetration, which is reminiscent of *mlo*-mediated resistance (see Sect. 11.6.2). This is different from nonhost resistance to the wheat leaf rust *P. triticina*: Here the fungus was either stopped at the pre-haustorial (penetration) phase or after the formation of the first haustorium (Neu et al. 2003). Lastly, the nonhost-resistant interaction with *M. grisea* was characterized by slightly enhanced formation of non-penetrated cell-wall appositions. Also, levels of epidermal cell death were similar to *M. oryzae* attack, but mesophyll cell death was completely abolished (Zellerhoff et al. 2006). Therefore, the most efficient block in the development of non-adapted *M. grisea* in barley occurred when the pathogen attempted to grow into the underlying mesophyll. It remains currently open if this reflects a late epidermal defence response, a lack of delivery of essential nutrients by the nonhost or the exclusive competence of mesophyll cells to stop fungal infection.

Transcript profiling has been performed to further characterize nonhost resistance of one barley genotype to powdery mildew, rust and blast fungi (Zellerhoff et al. 2010). A large overlap was observed between powdery mildew and rust, powdery mildew and blast and rust and blast interactions of those transcripts that were similarly regulated in both host and nonhost interactions. In contrast, overlap of those transcripts that were only significantly regulated during nonhost interactions showed little overlap between pathosystems. This gave rise to the model of a general PAMP-triggered transcriptional response, which however suffers from host pathogen-specific repression due to different sets of effectors released either by powdery mildew, rust or blast.

Some barley genes have been proposed to be functionally involved in nonhost resistance. So far, published evidence was derived from transient expression or TIGS experiments and will have to be confirmed at the level of stably transformed plants or TILLING mutants. Transient overexpression of barley *Mlo*, bax inhibitor (*HvBI-1*) or actin depolymerising factor (*HvADF3*) genes was found to weaken nonhost resistance to the wheat powdery mildew (Elliott et al. 2002; Huckelhoven et al. 2003; Miklis et al. 2007). TIGS of the syntaxin family protein *HvSNAP34* was found to enhanced nonhost susceptibility to wheat powdery mildew (Douchkov et al. 2005), as did 9 additional genes that came out from a TIGS screening of 725 upregulated transcripts in *B. graminis*-attacked epidermis (Douchkov et al. unpublished). These transgene effects are compatible with a model of nonhost resistance of barley to inappropriate powdery mildew fungi that includes the absence of defence (cell death?) suppression due to inefficient effectors on the one hand as well as efficient reorganization of cell polarity and secretion of defence-related compounds and proteins on the other.

11.6.2 Race-Nonspecific Host Resistance

Race-nonspecific host resistance (NR) is a likely manifestation of PTI and of high interest to barley breeders because it promises to provide an acceptable level of quantitive resistance over an extended period of time in the field (Jorgensen 1992). Successful examples of strong NR in barley or wheat caused by single key genes such as *mlo* (7-transmembrane domain protein), *Rpg1* (receptor kinase-like), *LR34* (ABC transporter) or *pm21* (protein kinase) indeed exist (Jorgensen 1992; Brueggeman et al. 2002; Krattinger et al. 2009; Cao et al. 2011). However, such strongly acting genes cannot always be identified, which leaves breeders with the option to combine useful QTL alleles, each one ideally explaining not less than 10 % of the observed phenotypic variation. To date no resistance QTL-underlying gene has been cloned in barley; and therefore, models explaining quantitative resistance mechanisms have remained speculative.

Nevertheless, there exists suggestive evidence for specific gene functional groups likely to be important for NR. In a candidate-gene study, peroxidases have been found to co-occupy recombination bins more abundantly with QTL for resistance to barley leaf rust and powdery mildew than expected by chance (Gonzalez et al. 2010). The same has been reported for secreted class III peroxidases, receptor-like protein kinases and factors of vesicle transport in *Bgh*-attacked barley (Schweizer and Stein 2011). Representatives of these functional groups of genes have recently gained considerable attention as key factors of PTI and include the flagellin receptor FLS2, t- and v-SNARE proteins and the peroxidases TaPrx103 and HvPrx40 to name just a few (Collins et al. 2003; Zipfel et al. 2004; Johrde and Schweizer 2008; Kwon et al. 2008; Schweizer 2008).

11.6.3 Race-Specific Host Resistance

On top of PTI ETI causes strong protection against fungal races that carry effector genes matching the recognition specificity of a corresponding NB-LRR-type *R*-gene. This type of resistance is inherited as a single Mendelian trait and thus is easy to handle in breeding practice (Jorgensen 1994). As discussed above its durability often is very limited thereby requiring a constant pipeline of novel *R*-genes in germplasm as additional burden to competitive breeding (Brown et al. 1993). However, durable, monogenic resistances acting in a race-specific manner against fungi do exist implying that a priori pessimism with regard to the usefulness of race-specific *R*-genes may not be appropriate. Typical examples of major *R*-genes usually exhibiting a high degree of fungal race specificity are the *Ml* genes including the *Mla1–Mla32* allelic series for resistance to *Bgh* (Jorgensen 1994), *Rph1–Rph19* for resistance to *P. hordei* (Weerasena et al. 2004) or *Rrs1–Rrs15* for resistance to *R. commune* (Wagner et al. 2008). In some interactions such as the one with *F. graminearum* or *B. sorokiniana*, no or very few *R*-genes mediating

qualitative and race-specific resistance have been identified until present but instead, small- to major-effect QTL were found (Kumar et al. 2002; Bai and Shaner 2004). So far one *R*-gene (*Mla*) was molecularly isolated encoding an NB LRR-type protein and therefore implicating ETI as underlying mechanism, as expected based on of a large body of data in other plant species (Zhou et al. 2001).

11.7 Improving Durable Barley Resistance

I see three principal ways to improve durable resistance of barley to major fungal pathogens: (1) stacking of carefully selected major R-genes by breeding, (2) - marker-assisted introgression of multiple QTL by breeding and (3) generation of transgenic events introducing novel resistance or defence genes derived from barley, wild *Hordeum* relatives or other plant species; or silencing of susceptibility factors.

11.7.1 Breeding

Race-specific major *R*-genes are often overcome in the field by new pathogen races within a short period of time, due to the ease of eliminating or modifying one out of a larger set of redundantly acting effector proteins. Although not a priori expected, even simultaneously introduced pairs of *R*-genes against the same pathogen were readily broken down (Brown et al. 1993). Therefore, in order to improve the durability of this type of resistance, more efforts are required. Especially, deeper knowledge about pathogen populations and effector functions would allow searching for and selecting *R*-genes that recognize highly conserved and (more) essential effectors. The successful identification of *R*-genes in tomato identifying highly conserved and essential Ecm effectors in *Cladosporium fulvum* has provided promising proof of concept for this effector-oriented approach (Lauge et al. 1998; Stergiopoulos et al. 2010). Stacking two *R*-genes of this category might provide a new level of resistance durability.

According to a large number of QTL studies in barley, robust quantitative resistance of a given donor is often inherited by two or more QTL. Efficient exploitation will therefore require a QTL-stacking approach. Of course, QTL stacking would be more interesting if genetic loci of meta-QTL against several diseases could be used, which might not be unrealistic based on a recent meta-QTL study in barley to all foliar diseases addressed here (Schweizer and Stein 2011). In addition to focussing on multi-interaction QTL, one could emphasize on potentially important genes or gene families that co-localize with the QTL of interest. By doing so in the meta-QTL study mentioned above, it was possible to identify, e.g. receptor-like protein kinases or secreted class III peroxidases as particularly promising candidates for gene marker development (Schweizer and Stein 2011).

Besides transcript regulation and QTL co-localization, candidate genes can also be assessed with respect to interaction phenotypes upon RNAi as well as the existence of resistance- or susceptibility-associated gene haplotypes or SNP. Combining these and possibly other datasets may create a body of converging evidence for the importance of specific genes in durable host resistance. This approach recently led to the identification of nine interesting barley gene candidates for future exploitation in translational research (Douchkov et al. 2011; Spies et al. 2012).

However, irrespective of the nature of the introgressed alleles by breeding, suppressed meiotic recombination frequency near centromeres and at loci of introgressed DNA from remotely related genotypes such as wild *Hordeum* species creates problems of linkage drag associated with undesirable traits that are difficult to cross out of the genome of the recurrent elite parent (Ruge-Wehling et al. 2006; Comadran et al. 2011).

11.7.2 Transgenic Approaches

A way out of the dilemma of linkage drag around interesting genes derived e.g. from wide crosses is the introgression of defines genes by gene transfer resulting in transgenic barley events. It has been shown in a large number of reports that gene technology is well suited for the introduction into crop plants of DNA sequences from the same or different species that can belong to highly unrelated taxa of the tree of life. In the latter case codon-usage optimization might be required for a sufficient level of expression. Efficient barley transformation protocols exist, especially for a small number of model cultivars (Hensel et al. 2008). Because the easiest transformable cv. Golden Promise used to be an elite malting barley in the sixties of the last century, eventual backcrossing into target cultivars is expected to produce less linkage drag problems compared to using exotic donor material. This leaves us with a situation where basically only human engineering spirit and the application potential of selected genes limits success.

A first promising transgenic approach to durable resistance is the introduction of major *R*-genes from highly resistant wild relatives of crop plants. Convincing proof of concept for this approach has been obtained in potato where two genes for resistance to the potato late blight pathogen *Phytophthora infestans* were introduced from the wild potato *S. bulbocastanum* (van der Vossen et al. 2003, 2005). Extensive field trials of the selected events over a number of years showed immunity to this devastating disease.

A second interesting approach is the silencing of susceptibility-related genes of barley. If successful, transgenic events would be released from effector-mediated defence suppression similar to the situation in *mlo* loss-of-function mutants showing immunity to *Bgh* (Piffanelli et al. 2002). Alternatively transgenic plants might also refuse to deliver nutrients to fungal pathogens, although this strategy will most likely be restricted to (hemi)biotrophic pathogens that are dependent on regulated active nutrient export from the host plant, at least during the early (biotrophic)

phase of the interaction. Promising target genes in this respect might be glutamate or aspartate transporters as well as SWEET sugar transporters localized in lipid raft-like membranes around haustoria (Chen et al. 2010a). We found function evidence by TIGS for an important role of barley SWEET proteins not only in supporting *Bgh* development but also in providing energy for defence reactions (Douchkov and Schweizer, unpublished). Other potentially interesting, susceptibility-related genes of barley encode bax inhibitor 1 or WRKY1-3 transcription factors. Indeed, transgenic barley carrying RNAi constructs against these targets showed clearly enhanced resistance to *Bgh* (Eichmann et al. 2010; Himmelbach, Gurushizde, Hensel, Schweizer and Kumlehn, unpublished).

A third approach worth mentioning in this context is host-induced gene silencing (HIGS) of essential housekeeping, cell wall-related or pathogenicity-related target genes of fungal pathogens. It was shown recently that fungal pathogens attacking corresponding transiently silenced or transgenic barley, wheat or tobacco plants are compromised in their development and exhibit silencing of the GUS reporter as well as endogenous target genes (Nowara et al. 2010; Tinoco et al. 2010; Yin et al. 2011). More work will have to be invested to test if this promising concept, which can only be realized in transgenic plants, might be suitable to provide strong resistance in the field. Durability of the engineered HIGS resistance traits will most likely be high because fungi are not expected to delete essential components of their gene-silencing machinery to escape HIGS. Moreover, single point mutations of HIGS target genes will have no effect because the introduced h.a.i.rpin constructs usually cover several hundred bp of fungal DNA, which will leave ample efficient siRNA molecules left and right from any eventual mutation.

Conclusion

Understanding how barley or any other crop plant responds to the array of relevant pathogens provides, together with a comprehensive set of identified major and minor resistance loci, a basis for knowledge-based improvement of durable resistance. Genome-wide transcript-profiling data are an attractive way of comparing host and nonhost responses across interactions but such meta-analyses are currently still largely missing. I therefore believe that the combination of meta-gene regulation data with meta-QTL analysis will provide lead genes for allele mining in genetically diverse, phenotyped barley populations stored in ex situ collections. Resistance-associated alleles would have subsequently to be introgressed into susceptible modern cultivars by using the genes as markers, and the probability of success of the gene-based strategy may be further increased by performing functional assays such as transient or stable gene silencing previous to backcrossing. On the medium to long run, the expected result from such an integrated approach will be a collection of lead genes for durable resistance of barley to important single or multiple fungal diseases.

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References

Able AJ (2003) Role of reactive oxygen species in the response of barley to necrotrophic pathogens. Protoplasma 221:137–143

Aghnoum R, Niks RE (2010) Specificity and levels of nonhost resistance to nonadapted Blumeria graminis forms in barley. New Phytol 185:275–284

An QL, Huckelhoven R, Kogel KH, Van Bel AJE (2006) Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. Cell Microbiol 8:1009–1019

Atienza SG, Jafary H, Niks RE (2004) Accumulation of genes for susceptibility to rust fungi for which barley is nearly a nonhost results in two barley lines with extreme multiple susceptibility. Planta 220:71–79

Avrova A, Knogge W (2012) Rhynchosporium commune: a persistent threat to barley cultivation. Mol Plant Pathol 13:986–997

Bai GH, Shaner G (2004) Management and resistance in wheat and barley to Fusarium head blight. Annu Rev Phytopathol 42:135–161

Babaeizad V, Imani J, Kogel KH, Eichmann R, Hückelhoven R (2009) Over-expression of the cell death regulator BAX inhibitor-1 in barley confers reduced or enhanced susceptibility to distinct fungal pathogens. Theor Appl Genet 118:455–463

Beccari G, Covarelli L, Nicholson P (2011) Infection processes and soft wheat response to root rot and crown rot caused by Fusarium culmorum. Plant Pathol 60:671–684

Bhat RA, Miklis M, Schmelzer E, Schulze-Lefert P, Panstruga R (2005) Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. Proc Natl Acad Sci U S A 102:3135–3140

Boddu J, Cho S, Kruger WM, Muehlbauer GJ (2006) Transcriptome analysis of the barley-Fusarium graminearum interaction. Mol Plant Microbe Interact 19:407–417

Boddu J, Cho SH, Muehlbauer GJ (2007) Transcriptome analysis of trichothecene-induced gene expression in barley. Mol Plant Microbe Interact 20:1364–1375

Bogacki P, Oldach KH, Williams KJ (2008) Expression profiling and mapping of defence response genes associated with the barley-Pyrenophora teres incompatible interaction. Mol Plant Pathol 9:645–660

Bollina V, Kushalappa AC, Choo TM, Dion Y, Rioux S (2011) Identification of metabolites related to mechanisms of resistance in barley against Fusarium graminearum, based on mass spectrometry. Plant Mol Biol 77:355–370

Brown JKM, Simpson CG, Wolfe MS (1993) Adaptation of barley powdery mildew populations in England to varieties with 2 resistance genes. Plant Pathol 42:108–115

Brown NA, Urban M, Van De Meene AML, Hammond-Kosack KE (2010) The infection biology of Fusarium graminearum: defining the pathways of spikelet to spikelet colonisation in wheat ears. Fungal Biol 114:555–571

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B, Kleinhofs A (2002) The barley stem rust-resistance gene Rpg1 is a novel disease-resistance gene with homology to receptor kinases. Proc Natl Acad Sci U S A 99:9328–9333

Brueggeman R, Druka A, Nirmala J, Cavileer T, Drader T, Rostoks N, Mirlohi A, Bennypaul H, Gill U, Kudrna D, Whitelaw C, Kilian A, Han F, Sunl Y, Gill K, Steffenson B, Kleinhofs A

(2008) The stem rust resistance gene Rpg5 encodes a protein with nucleotide-binding-site, leucine-rich, and protein kinase domains. Proc Natl Acad Sci U S A 105:14970–14975
Caldo RA, Nettleton D, Wise RP (2004) Interaction-dependent gene expression in Mla-specified response to barley powdery mildew. Plant Cell 16:2514–2528
Caldo RA, Nettleton D, Peng JQ, Wise RP (2006) Stage-specific suppression of basal defense discriminates barley plants containing fast- and delayed-acting Mla powdery mildew resistance alleles. Mol Plant Microbe Interact 19:939–947
Cao AH, Xing LP, Wang XY, Yang XM, Wang W, Sun YL, Qian C, Ni JL, Chen YP, Liu DJ, Wang X, Chen PD (2011) Serine/threonine kinase gene Stpk-V, a key member of powdery mildew resistance gene Pm21, confers powdery mildew resistance in wheat. Proc Natl Acad Sci U S A 108:7727–7732
Chen LQ, Hou BH, Lalonde S, Takanaga H, Hartung ML, Qu XQ, Guo WJ, Kim JG, Underwood W, Chaudhuri B, Chermak D, Antony G, White FF, Somerville SC, Mudgett MB, Frommer WB (2010a) Sugar transporters for intercellular exchange and nutrition of pathogens. Nature 468:527–532
Chen XW, Niks RE, Hedley PE, Morris J, Druka A, Marcel TC, Vels A, Waugh R (2010b) Differential gene expression in nearly isogenic lines with QTL for partial resistance to Puccinia hordei in barley. BMC Genomics 11:629
Choo TM, Martin RA, Ho KM, Shen Q, Fedak G, Savard M, Voldeng H, Falk DE, Etienne M, Sparry E (2004) Fusarium head blight and deoxynivalenol accumulation of barley in eastern canada: cultivar response and correlation analysis. Plant Dis 88:837–844
Colebrook EH, Creissen G, McGrann GRD, Dreos R, Lamb C, Boyd LA (2012) Broad-spectrum acquired resistance in barley induced by the Pseudomonas pathosystem shares transcriptional components with Arabidopsis systemic acquired resistance. Mol Plant Microbe Interact 25:658–667
Collinge DB (2009) Cell wall appositions: the first line of defence. J Exp Bot 60:351–352
Collinge DB, Jorgensen HJL, Lund OS, Lyngkjaer MF (2010) Engineering pathogen resistance in crop plants: current trends and future prospects. Annu Rev Phytopathol 48:269–291
Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Huckelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425:973–977
Comadran J, Ramsay L, MacKenzie K, Hayes P, Close TJ, Muehlbauer G, Stein N, Waugh R (2011) Patterns of polymorphism and linkage disequilibrium in cultivated barley. Theor Appl Genet 122:523–531
Douchkov D, Nowara D, Zierold U, Schweizer P (2005) A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells. Mol Plant Microbe Interact 18:755–761
Douchkov D, Johrde A, Nowara D, Himmelbach A, Lueck S, Niks R, Schweizer P (2011) Convergent evidence for a role of WIR1 proteins during the interaction of barley with the powdery mildew fungus Blumeria graminis. J Plant Physiol 168:20–29
Eichmann R, Schultheiss H, Kogel KH, Huckelhoven R (2004) The barley apoptosis suppressor homologue bax inhibitor-1 compromises nonhost penetration resistance of barley to the inappropriate pathogen Blumeria graminis f. sp tritici. Mol Plant Microbe Interact 17:484–490
Eichmann R, Bischof M, Weis C, Shaw J, Lacomme C, Schweizer P, Duchkov D, Hensel G, Kumlehn J, Huckelhoven R (2010) BAX INHIBITOR-1 is required for full susceptibility of barley to powdery mildew. Mol Plant Microbe Interact 23:1217–1227
Elliott C, Zhou FS, Spielmeyer W, Panstruga R, Schulze-Lefert P (2002) Functional conservation of wheat and rice Mlo orthologs in defense modulation to the powdery mildew fungus. Mol Plant Microbe Interact 15:1069–1077
Felle HH, Herrmann A, Schaefer P, Hueckelhoven R, Kogel KH (2008) Interactive signal transfer between host and pathogen during successful infection of barley leaves by Blumeria graminis and Bipolaris sorokiniana. J Plant Physiol 165:52–59

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–227

Gonzalez AM, Marcel TC, Kohutova Z, Stam P, van der Linden CG, Niks RE (2010) Peroxidase profiling reveals genetic linkage between Peroxidase gene clusters and basal host and non-host resistance to rusts and mildew in barley. PLoS One 5:e10495

Goswami RS, Kistler HC (2004) Heading for disaster: Fusarium graminearum on cereal crops. Mol Plant Pathol 5:515–525

Gregersen PL, Thordal-Christensen H, Forster H, Collinge DB (1997) Differential gene transcript accumulation in barley leaf epidermis and mesophyll in response to attack by Blumeria graminis f.sp. hordei (syn. Erysiphe graminis f.sp. hordei). Physiol Mol Plant Pathol 51:85–97

Hahn M, Juengling S, Knogge W (1993) Cultivar-specific elicitation of barley defense reactions by the phytotoxic peptide NIP1 from Rhynchosporium secalis. Mol Plant Microbe Interact 6:745–754

Hallen-Adams HE, Wenner N, Kuldau GA, Trail F (2011) Deoxynivalenol biosynthesis-related gene expression during wheat kernel colonization by Fusarium graminearum. Phytopathology 101:1091–1096

Hensel G, Valkov V, Middlefell-Williams J, Kumlehn J (2008) Efficient generation of transgenic barley: the way forward to modulate plant-microbe interactions. J Plant Physiol 165:71–82

Hoefle C, Huesmann C, Schultheiss H, Bornke F, Hensel G, Kumlehn J, Huckelhoven R (2011) A barley ROP GTPase ACTIVATING PROTEIN associates with microtubules and regulates entry of the barley powdery mildew fungus into leaf epidermal cells. Plant Cell 23:2422–2439

Horbach R, Navarro-Quesada AR, Knogge W, Deising HB (2011) When and how to kill a plant cell: infection strategies of plant pathogenic fungi. J Plant Physiol 168:51–62

Huang K, Czymmek KJ, Caplan JL, Sweigard JA, Donofrio NM (2011) HYR1-mediated detoxification of reactive oxygen species is required for full virulence in the rice blast fungus. PLoS Pathog 7:e1001335

Huckelhoven R (2007) Cell wall – associated mechanisms of disease resistance and susceptibility. Annu Rev Phytopathol 45:101–127

Huckelhoven R, Kogel KH (1998) Tissue-specific superoxide generation at interaction sites in resistant and susceptible near-isogenic barley lines attacked by the powdery mildew fungus (Erysiphe graminis f. sp. hordei). Mol Plant Microbe Interact 11:292–300

Huckelhoven R, Panstruga R (2011) Cell biology of the plant-powdery mildew interaction. Curr Opin Plant Biol 14:738–746

Huckelhoven R, Fodor J, Preis C, Kogel KH (1999) Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. Plant Physiol 119:1251–1260

Huckelhoven R, Dechert C, Kogel KH (2003) Overexpression of barley BAX inhibitor 1 induces breakdown of mlo-mediated penetration resistance to Blumeria graminis. Proc Natl Acad Sci U S A 100:5555–5560

Huesmann C, Reiner T, Hoefle C, Preuss J, Jurca ME, Domoki M, Feher A, Huckelhoven R (2012) Barley ROP binding kinase1 is involved in microtubule organization and in basal penetration resistance to the barley powdery mildew fungus. Plant Physiol 159:311–320

Jafary H, Szabo LJ, Niks RE (2006) Innate nonhost immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities. Mol Plant Microbe Interact 19:1270–1279

Jafary H, Albertazzi G, Marcel TC, Niks RE (2008) High diversity of genes for nonhost resistance of barley to heterologous rust fungi. Genetics 178:2327–2339

Jansen C, von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proc Natl Acad Sci U S A 102:16892–16897

Jarosch B, Kogel KH, Schaffrath U (1999) The ambivalence of the barley Mlo locus: mutations conferring resistance against powdery mildew (Blumeria graminis f. sp, hordei) enhance

susceptibility to the rice blast fungus Magnaporthe grisea. Mol Plant Microbe Interact 12:508–514
Jarosch B, Collins NC, Zellerhoff N, Schaffrath U (2005) RAR1, ROR1, and the actin cytoskeleton contribute to basal resistance to Magnaporthe grisea in barley. Mol Plant Microbe Interact 18:397–404
Jensen MK, Rung JH, Gregersen PL, Gjetting T, Fuglsang AT, Hansen M, Joehnk N, Lyngkjaer MF, Collinge DB (2007) The HvNAC6 transcription factor: a positive regulator of penetration resistance in barley and Arabidopsis. Plant Mol Biol 65:137–150
Johrde A, Schweizer P (2008) A class III peroxidase specifically expressed in pathogen-attacked barley epidermis contributes to basal resistance. Mol Plant Pathol 9:687–696
Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329
Jorgensen JH (1992) Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. Euphytica 63:141–152
Jorgensen JH (1994) Genetics of powdery mildew resistance in barley. Crit Rev Plant Sci 13:97–119
Jorgensen HJL, Lubeck PS, Thordal-Christensen H, de Neergaard E, Smedegaard-Petersen V (1998) Mechanisms of induced resistance in barley against Drechslera teres. Phytopathology 88:698–707
Kale SD, Gu BA, Capelluto DGS, Dou DL, Feldman E, Rumore A, Arredondo FD, Hanlon R, Fudal I, Rouxel T, Lawrence CB, Shan WX, Tyler BM (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142:284–295
Kazan K, Gardiner DM, Manners JM (2012) On the trail of a cereal killer: recent advances in Fusarium graminearum pathogenomics and host resistance. Mol Plant Pathol 13:399–413
Khang CH, Berruyer R, Giraldo MC, Kankanala P, Park SY, Czymmek K, Kang S, Valent B (2010) Translocation of Magnaporthe oryzae effectors into rice cells and their subsequent cell-to-cell movement. Plant Cell 22:1388–1403
Kobayashi I, Kobayashi Y, Yamaoka N, Kunoh H (1992) Recognition of a pathogen and a nonpathogen by barley coleoptile cells. III. Responses of microtubules and actin-filaments in barley coleoptile cells to penetration attempts. Can J Bot 70:1815–1823
Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL, Keller B (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323:1360–1363
Kumar J, Huckelhoven R, Beckhove U, Nagarajan S, Kogel KH (2001) A compromised Mlo pathway affects the response of barley to the necrotrophic fungus Bipolaris sorokiniana (Teleomorph: Cochliobolus sativus) and its toxins. Phytopathology 91:127–133
Kumar J, Schafer P, Huckelhoven R, Langen G, Baltruschat H, Stein E, Nagarajan S, Kogel KH (2002) Bipolaris sorokiniana, a cereal pathogen of global concern: cytological and molecular approaches towards better control. Mol Plant Pathol 3:185–195
Kumaraswamy GK, Kushalappa AC, Choo TM, Dion Y, Rioux S (2012) Differential metabolic response of barley genotypes, varying in resistance, to trichothecene-producing and nonproducing (tri5-) isolates of Fusariam graminearum. Plant Pathol 61:509–521
Kwon C, Neu C, Pajonk S, Yun HS, Lipka U, Humphry M, Bau S, Straus M, Kwaaitaal M, Rampelt H, El Kasmi F, Jurgens G, Parker J, Panstruga R, Lipka V, Schulze-Lefert P (2008) Co-option of a default secretory pathway for plant immune responses. Nature 451:835–840
Lauge R, Joosten MH, Haanstra JP, Goodwin PH, Lindhout P, De Wit PJ (1998) Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen. Proc Natl Acad Sci U S A 95:9014–9018
Leisova-Svobodova L, Hanzalova A, Kucera L (2010) Expansion and variability of the Ptr Tox A gene in populations of Pyrenophora tritici-repentis and Pyrenophora teres. J Plant Pathol 92:729–735
Lim LG, Gaunt RE (1986) The effect of powdery mildew (Erysiphe-Graminis F-Sp Hordei) and leaf rust (Puccinia-Hordei) on spring barley in New-Zealand. I. Epidemic development, green leaf-area and yield. Plant Pathol 35:44–53

Linsell KJ, Keiper FJ, Forgan A, Oldach KH (2011) New insights into the infection process of Rhynchosporium secalis in barley using GFP. Fungal Genet Biol 48:124–131
Liu ZH, Ellwood SR, Oliver RP, Friesen TL (2011) Pyrenophora teres: profile of an increasingly damaging barley pathogen. Mol Plant Pathol 12:1–19
Manning VA, Hardison LK, Ciuffetti LM (2007) Ptr ToxA interacts with a chloroplast-localized protein. Mol Plant Microbe Interact 20:168–177
Marcel TC, Varshney RK, Barbieri M, Jafary H, de Kock MJD, Graner A, Niks RE (2007) A high-density consensus map of barley to compare the distribution of QTLs for partial resistance to Puccinia hordei and of defence gene homologues. Theor Appl Genet 114:487–500
Marcel TC, Gorguet B, Ta MT, Kohutova Z, Vels A, Niks RE (2008) Isolate specificity of quantitative trait loci for partial resistance of barley to Puccinia hordei confirmed in mapping populations and near-isogenic lines. New Phytol 177:743–755
McDonald BA, Linde C (2002) The population genetics of plant pathogens and breeding strategies for durable resistance. Euphytica 124:163–180
Meng Y, Wise RP (2012) HvWRKY10, HvWRKY19, and HvWRKY28 regulate Mla-triggered immunity and basal defense to barley powdery mildew. Mol Plant Microbe Interact 25:1492–1505
Miethbauer S, Heiser I, Liebermann B (2003) The phytopathogenic fungus Ramularia collo-cygni produces biologically active rubellins on infected barley leaves. J Phytopathol 151:665–668
Miklis M, Consonni C, Bhat RA, Lipka V, Schulze-Lefert P, Panstruga R (2007) Barley MLO modulates actin-dependent and actin-independent antifungal defense pathways at the cell periphery. Plant Physiol 144:1132–1143
Millett BP, Xiong YW, Dahl SK, Steffenson BJ, Muehlbauer GJ (2009) Wild barley accumulates distinct sets of transcripts in response to pathogens of different trophic lifestyles. Physiol Mol Plant Pathol 74:91–98
Moscou MJ, Lauter N, Caldo RA, Nettleton D, Wise RP (2011a) Quantitative and temporal definition of the Mla transcriptional regulon during barley-powdery mildew interactions. Mol Plant Microbe Interact 24:694–705
Moscou MJ, Lauter N, Steffenson B, Wise RP (2011b) Quantitative and qualitative stem rust resistance factors in barley are associated with transcriptional suppression of defense regulons. PLoS Genet 7:e1002208
Neu C, Keller B, Feuillet C (2003) Cytological and molecular analysis of the Hordeum vulgare-Puccinia triticina nonhost interaction. Mol Plant Microbe Interact 16:626–633
Niks RE (1983a) Comparative histology of partial resistance and the non-host reaction to leaf rust pathogens in barley and wheat seedlings. Phytopathology 73:60–64
Niks RE (1983b) Haustorium formation by Puccinia-Hordei in leaves of hypersensitive, partially resistant, and non-host plant genotypes. Phytopathology 73:64–66
Niks RE, Rubiales D (2002) Potentially durable resistance mechanisms in plants to specialised fungal pathogens. Euphytica 124:201–216
Nowara D, Gay A, Lacomme C, Shaw J, Ridout C, Douchkov D, Hensel G, Kumlehn J, Schweizer P (2010) HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. Plant Cell 22:3130–3141
Oliveira P, Mauch A, Jacob F, Arendt EK (2012) Impact of Fusarium culmorum-infected barley malt grains on brewing and beer quality. J Am Soc Brew Chem 70:186–194
Papadopoulou-Bouraoui A, Vrabcheva T, Valzacchi S, Stroka J, Anklam E (2004) Screening survey of deoxynivalenol in beer from the European market by an enzyme-linked immunosorbent assay. Food Addit Contam 21:607–617
Parker D, Beckmann M, Zubair H, Enot DP, Caracuel-Rios Z, Overy DP, Snowdon S, Talbot NJ, Draper J (2009) Metabolomic analysis reveals a common pattern of metabolic re-programming during invasion of three host plant species by Magnaporthe grisea. Plant J 59:723–737
Pathuri IP, Zellerhoff N, Schaffrath U, Hensel G, Kumlehn J, Kogel KH, Eichmann R, Huckelhoven R (2008) Constitutively activated barley ROPs modulate epidermal cell size, defense reactions and interactions with fungal leaf pathogens. Plant Cell Rep 27:1877–1887

Persson M, Falk A, Dixelius C (2009) Studies on the mechanism of resistance to Bipolaris sorokiniana in the barley lesion mimic mutant bst1. Mol Plant Pathol 10:587–598

Pestka JJ (2007) Deoxynivalenol: toxicity, mechanisms and animal health risks. Anim Feed Sci Technol 137:283–298

Petti C, Reiber K, Ali SS, Berney M, Doohan FM (2012) Auxin as a player in the biocontrol of Fusarium head blight disease of barley and its potential as a disease control agent. BMC Plant Biol 12:224

Piffanelli P, Zhou FS, Casais C, Orme J, Jarosch B, Schaffrath U, Collins NC, Panstruga R, Schulze-Lefert P (2002) The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. Plant Physiol 129:1076–1085

Placinta CM, D'Mello JPF, Macdonald AMC (1999) A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. Anim Feed Sci Technol 78:21–37

Proels RK, Oberhollenzer K, Pathuri IP, Hensel G, Kumlehn J, Huckelhoven R (2010) RBOHF2 of barley is required for normal development of penetration resistance to the parasitic fungus Blumeria graminis f. sp hordei. Mol Plant Microbe Interact 23:1143–1150

Rayapuram C, Jensen MK, Maiser F, Shanir JV, Hornshoj H, Rung JH, Gregersen PL, Schweizer P, Collinge DB, Lyngkjaer MF (2012) Regulation of basal resistance by a powdery mildew-induced cysteine-rich receptor-like protein kinase in barley. Mol Plant Pathol 13:135–147

Rohe M, Gierlich A, Hermann H, Hahn M, Schmidt B, Rosahl S, Knogge W (1995) The race-specific elicitor, NIP1, from the barley pathogen, *Rhynchosporium secalis*, determines avirulence on host plants of the Rrs1 resistance genotype. EMBO J 14:4168–4177

Ruge-Wehling B, Linz A, Habekuss A, Wehling P (2006) Mapping of Rym16(Hb), the second soil-borne virus-resistance gene introgressed from Hordeum bulbosum. Theor Appl Genet 113:867–873

Schmidt SM, Panstruga R (2007) Cytoskeleton functions in plant-microbe interactions. Physiol Mol Plant Pathol 71:135–148

Schultheiss H, Dechert C, Kogel KH, Huckelhoven R (2003) Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus. Plant J 36:589–601

Schultheiss H, Hensel G, Imani J, Broeders S, Sonnewald U, Kogel KH, Kumlehn J, Huckelhoven R (2005) Ectopic expression of constitutively activated RACB in barley enhances susceptibility to powdery mildew and abiotic stress. Plant Physiol 139:353–362

Schultheiss H, Preuss J, Pircher T, Eichmann R, Huckelhoven R (2008) Barley RIC171 interacts with RACB in planta and supports entry of the powdery mildew fungus. Cell Microbiol 10:1815–1826

Schulze-Lefert P, Panstruga R (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. Trends Plant Sci 16:117–125

Schwarz PB, Beattie S, Casper HH (1996) Relationship between Fusarium infestation of barley and the gushing potential of malt. J Inst Brew 102:93–96

Schweizer P (2008) Tissue-specific expression of a defence-related peroxidase in transgenic wheat potentiates cell death in pathogen-attacked leaf epidermis. Mol Plant Pathol 9:45–57

Schweizer P, Stein N (2011) Large-scale data integration reveals colocalization of gene functional groups with meta-QTL for multiple disease resistance in barley. Mol Plant Microbe Interact 24:1492–1501

Shen QH, Saijo Y, Mauch S, Biskup C, Bieri S, Keller B, Seki H, Ulker B, Somssich IE, Schulze-Lefert P (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science 315:1098–1103

Skadsen RW, Hohn TA (2004) Use of Fusarium graminearum transformed with gfp to follow infection patterns in barley and Arabidopsis. Physiol Mol Plant Pathol 64:45–53

Spies A, Korzun L, Bayles R, Rajaraman J, Himmelbach A, Hedley PE, Schweizer P (2012) Allele mining in barley genetic resources reveals genes of race-nonspecific powdery mildew resistance. Front Plant Sci 2:113

Sreenivasulu N, Usadel B, Winter A, Radchuk V, Scholz U, Stein N, Weschke W, Strickert M, Close TJ, Stitt M, Graner A, Wobus U (2008) Barley grain maturation and germination: metabolic pathway and regulatory network commonalities and differences highlighted by new MapPlan/PageMan profiling tools. Plant Physiol 146:1738–1758

Stabentheiner E, Minihofer T, Huss H (2009) Infection of barley by Ramularia collo-cygni: scanning electron microscopic investigations. Mycopathologia 168:135–143

Steffenson BJ, Jin Y, Brueggeman RS, Kleinhofs A, Sun Y (2009) Resistance to stem rust race TTKSK Maps to the rpg4/Rpg5 complex of chromosome 5H of barley. Phytopathology 99:1135–1141

Steiner-Lange S, Fischer A, Boettcher A, Rouhara I, Liedgens H, Schmelzer E, Knogge W (2003) Differential defense reactions in leaf tissues of barley in response to infection by Rhynchosporium secalis and to treatment with a fungal avirulence gene product. Mol Plant Microbe Interact 16:893–902

Stergiopoulos I, van den Burg HA, Okmen B, Beenen HG, van Liere S, Kema GHJ, de Wit P (2010) Tomato Cf resistance proteins mediate recognition of cognate homologous effectors from fungi pathogenic on dicots and monocots. Proc Natl Acad Sci U S A 107:7610–7615

Tanaka S, Ichikawa A, Yamada K, Tsuji G, Nishiuchi T, Mori M, Koga H, Nishizawa Y, O'Connell R, Kubo Y (2010) HvCEBiP, a gene homologous to rice chitin receptor CEBiP, contributes to basal resistance of barley to Magnaporthe oryzae. BMC Plant Biol 10:288

Thirugnanasambandam A, Wright KM, Havis N, Whisson SC, Newton AC (2011) Agrobacterium-mediated transformation of the barley pathogen Ramularia collo-cygni with fluorescent marker tags and live tissue imaging of infection development. Plant Pathol 60:929–937

ThordalChristensen H, Zhang ZG, Wei YD, Collinge DB (1997) Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 11:1187–1194

Thordal-Christensen H, Gregersen PL, Collinge DB (eds) (2000) The barley/*Blumeria* (syn. *Erysiphe*) *graminis* interaction: a case study. Kluwer, Dordrecht

Tinoco MLP, Dias BBA, Dall'Astta RC, Pamphile JA, Aragao FJL (2010) In vivo trans-specific gene silencing in fungal cells by in planta expression of a double-stranded RNA. BMC Biol 8:27

Trujillo M, Kogel KH, Huckelhoven R (2004) Superoxide and hydrogen peroxide play different roles in the nonhost interaction of barley and wheat with inappropriate formae speciales of Blumeria graminis. Mol Plant Microbe Interact 17:304–312

Urashima AS, Igarashi S, Kato H (1993) Host-range, mating-type, and fertility of Pyricularia-grisea from wheat in brazil. Plant Dis 77:1211–1216

van der Vossen E, Sikkema A, Hekkert BTL, Gros J, Stevens P, Muskens M, Wouters D, Pereira A, Stiekema W, Allefs S (2003) An ancient R gene from the wild potato species Solanum bulbocastanum confers broad-spectrum resistance to Phytophthora infestans in cultivated potato and tomato. Plant J 36:867–882

van der Vossen EAG, Gros J, Sikkema A, Muskens M, Wouters D, Wolters P, Pereira A, Allefs S (2005) The Rpi-blb2 gene from Solanum bulbocastanum is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato. Plant J 44:208–222

Wagacha JM, Muthomi JW (2007) Fusarium culmorum: infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. Crop Protect 26:877–885

Wagner C, Schweizer G, Kramer M, Dehmer-Badani A, Ordon F, Friedt W (2008) The complex quantitative barley-Rhynchosporium secalis interaction: newly identified QTL may represent already known resistance genes. Theor Appl Genet 118:113–122

Walters DR, Havis ND, Oxley SJP (2008) Ramularia collo-cygni: the biology of an emerging pathogen of barley. FEMS Microbiol Lett 279:1–7

Wang CF, Huang LL, Zhang HC, Han QM, Buchenauer H, Kang ZS (2010) Cytochemical localization of reactive oxygen species (O-2(-) and H2O2) and peroxidase in the incompatible

and compatible interaction of wheat – Puccinia striiformis f. sp tritici. Physiol Mol Plant Pathol 74:221–229

Ward R (2007) The global threat posed by Ug99. Phytopathology 97:S136

Weerasena JS, Steffenson BJ, Falk AB (2004) Conversion of an amplified fragment length polymorphism marker into a co-dominant marker in the mapping of the Rph15 gene conferring resistance to barley leaf rust, Puccinia hordei Otth. Theor Appl Genet 108:712–719

Wevelsiep L, Kogel KH, Knogge W (1991) Purification and characterization of peptides from Rhynchosporium-secalis inducing necrosis in barley. Physiol Mol Plant Pathol 39:471–482

Wevelsiep L, Rupping E, Knogge W (1993) Stimulation of barley plasmalemma H+-ATPase by phytotoxic peptides from the fungal pathogen Rhynchosporium-secalis. Plant Physiol 101:297–301

Wise RP, Lauter N, Szabo LJ, Schweizer P (2009) Genomics of biotic interactions in the Triticeae. In: Muehlbauer GJ, Feuillet C (eds) Genetics and genomics of the Triticeae, vol 7. Springer, New York, NY, pp 559–589

Yang F, Jensen JD, Svensson B, Jorgensen HJL, Collinge DB, Finnie C (2010) Analysis of early events in the interaction between Fusariam graminearum and susceptible barley (Hordeum vulgare) cultivar Scarlett. Proteomics 10:3748–3755

Yang F, Jensen JD, Svensson B, Jorgensen HJL, Collinge DB, Finnie C (2012) Secretomics identifies Fusarium graminearum proteins involved in the interaction with barley and wheat. Mol Plant Pathol 13:445–453

Yin CT, Jurgenson JE, Hulbert SH (2011) Development of a host-induced RNAi system in the wheat stripe rust fungus Puccinia striiformis f. sp tritici. Mol Plant Microbe Interact 24:554–561

Zellerhoff N, Jarosch B, Groenewald JZ, Crous PW, Schaffrath U (2006) Nonhost resistance of barley is successfully manifested against Magnaporthe grisea and a closely related Pennisetum-infecting lineage but is overcome by Magnaporthe oryzae. Mol Plant Microbe Interact 19:1014–1022

Zellerhoff N, Jansen M, Schaffrath U (2008) Barley Rom1 antagonizes Rar1 function in Magnaporthe oryzae-infected leaves by enhancing epidermal and diminishing mesophyll defence. New Phytol 180:702–710

Zellerhoff N, Himmelbach A, Dong WB, Bieri S, Schaffrath U, Schweizer P (2010) Nonhost resistance of barley to different fungal pathogens is associated with largely distinct, quantitative transcriptional responses. Plant Physiol 152:2053–2066

Zhang L, Lavery L, Gill U, Gill K, Steffenson B, Yan GP, Chen XM, Kleinhofs A (2009) A cation/proton-exchanging protein is a candidate for the barley NecS1 gene controlling necrosis and enhanced defense response to stem rust. Theor Appl Genet 118:385–397

Zhou FS, Kurth JC, Wei FS, Elliott C, Vale G, Yahiaoui N, Keller B, Somerville S, Wise R, Schulze-Lefert P (2001) Cell-autonomous expression of barley Mla1 confers race-specific resistance to the powdery mildew fungus via a Rar1-independent signaling pathway. Plant Cell 13:337–350

Zierold U, Scholz U, Schweizer P (2005) Transcriptome analysis of mlo-mediated resistance in the epidermis of barley. Mol Plant Pathol 6:139–151

Zimmermann G, Baumlein H, Mock HP, Himmelbach A, Schweizer P (2006) The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. Plant Physiol 142:181–192

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428:764–767

Chapter 12
Responses to Phytophagous Arthropods

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12.1 Introduction

Barley (*Hordeum vulgare L.*) as other cereal species is targeted by different pests causing significant damages when the environmental and cultural conditions are favourable for their development. Although damage from field insects is not a major hazard for barley crops, this chapter summarises the main insect species considered as a threat of barley growth and seed yield, as well as the physical barriers and chemical compounds developed by *Hordeum* genotypes to combat insect attack, particularly aphid pests. New insights into the molecular mechanisms of barley–pest interactions and the use of novel molecular approaches are discussed. Finally, the integration of candidate barley genes with defence properties into plant genome to generate resistance against pest opens up future conventional plant-assessment programmes.

12.2 Barley Pests

This section deals with phytophagous arthropods affecting barley. It includes only those species that are considered a serious threat for grain quality and yield losses. Damage in growing barley plants from field pests can be a limiting factor for this cereal and, in some cases such as aphid or mite species, may cause additional secondary relevant effects as virus vectors. Additionally, stored barley grains

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infested with seed beetles, borers or moths produce a reduction of their nutritive value and germination capacity and subsequently dropping grain sales and/or exportation.

Among the barley-associated pests, there are several damaging aphids (Homoptera) that can be found from emergence to harvest. Aphids feed on barley leaves by inserting their stylets into the phloem and sucking plant sap. Probably, the most detrimental are the Russian wheat aphid, *Diuraphis noxia,* which gives rise to leaf rolling and discolouration and elicits significant stunting of seedlings, and the greenbug, *Schizaphis graminum,* which leads to yellow or red leaf spots and ends to typical leaf yellowing and eventually necrosis. Population dynamics of *S. graminum,* especially biotypic variations on cereals and noncultivated grasses, have been studied. These studies have allowed the recognition of different biotypes that damage small grains (Weng et al. 2010; Shufran 2011). Besides, the bird cherry-oat aphid, *Rhopalosiphum padi,* does not produce specific symptoms but causes plant root and shoot reductions that lead to yield losses at high population densities, and it is an important pest and a primary vector of barley yellow dwarf viruses (Fig. 12.1). In addition, the grain aphid, *Sitobion avenae,* is also a usual cause of direct injury to spring barley crops (http://www.plantprotection.hu/modulok/angol/barley/aphids_bar.htm).

Recently, the cereal leaf beetle, *Oulema melanopus* (Coleoptera, Chrysomelidae), has turn into an important pest of cereals, mainly in North America. Both adult and larvae beetles are voracious feeders that damage plants by chewing out long strips of tissue between veins of the barley leaves. When damage is extensive, the leaves turn whitish and the plants appear as if injured by frost.

Regarding the stored grain pests, the most common pests found in barley include the weevils *Sitophilus oryzae* and *S. granarius* (Coleoptera, Curculionidae), the rust-red flour beetle *Tribolium castaneum* (Coleoptera, Tenebrionidae) and the Angoumois grain moth *Sitotroga cerealella* (Lepidoptera, Gelechiidae). Primary granary infestations are produced by chewing insects that feed on whole grains causing significant reduction in seed viability. Subsequently, other chewing species recognised as secondary pests of grain increase the feeding damage caused by primary pests (Mozos-Pascual 1997). Eventually, severe pest infestations generate grain contamination by insect dead bodies, cast skins and faecal pellets and sometimes induce unfavourable changes in chemical composition of the grain or even could be the origin of allergic reactions.

As in other plant species, there is an increased demand of resistant barley genotypes to combat pests, particularly related to aphid resistance. In the case of *D. noxia* and *S. graminum*, resistant barley germ plasm has been successfully identified based on reduced plant symptoms, where single genes with large effects on resistance have been identified (Gardenhire 1979; Webster et al. 1991). Conversely, to determine the resistance levels to *R. padi* is more difficult due to the absence of visible symptoms produced by this aphid in barley plants (Saheed et al. 2007). The selection of resistant *Hordeum* genotypes to *R. padi* has been based on differential aphid responses and behaviour (Ahman et al. 2000). Only

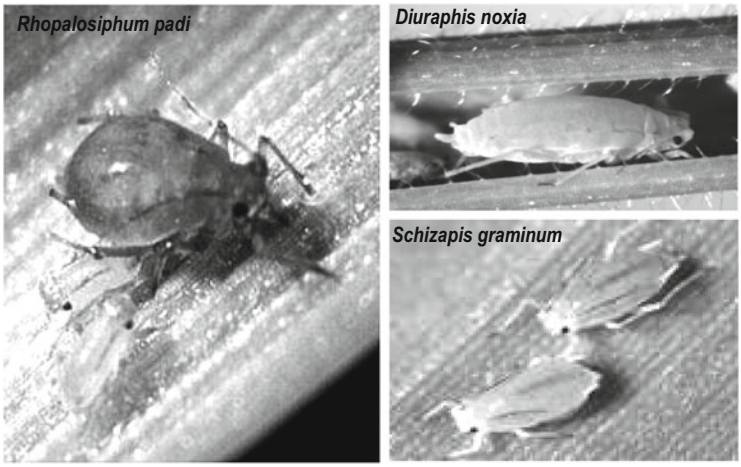

Fig. 12.1 Most common barley aphid species. The Russian wheat aphid, *Diuraphis noxia;* the greenbug, *Schizaphis graminum;* and the bird cherry-oat aphid, *Rhopalosiphum padi*

partial resistance controlled by a number of unspecified genes has been found in barley against *R. padi*, being therefore quite difficult to handle in plant breeding programmes (Porter et al. 1999).

Cereals have also a role as primary hosts to spread infections to other crops. This is the case of the polyphagous green peach aphid, *Myzus persicae*, which is an important vector of potato-infecting viruses (Davis and Radcliffe 2008). The use of barrier crops is an alternative strategy to protect potato-production fields. Cereals are targets of cereal aphids which do not colonise potato. The greater abundance of these less efficient vector species can make them more important in potato Y virus epidemiology.

12.3 Differential Defence Responses to Pest Among Barley Genotypes

Plants are immobile organisms that cannot escape from unfavourable situations as pest attack but are able to develop an arsenal of physical and chemical defences to protect themselves. Similarities in the general plant responses to different pest feeding and unique responses to specific pest plant interaction in pest-resistant plants have been described. Saheed et al. (2007) showed that the different symptoms produced after *D. noxia* and *R. padi* infestation in plants are due to different effects on their host, including barley. The Russian wheat aphid deposits dense saliva which encases the inner walls of affected xylem elements causing severe damage of phloem tissues, whereas xylem tapped by bird cherry-oat aphids contains more granular saliva, which apparently does not occlude vessel wall apertures.

Moreover, many effectors that modulate plant defences have been found in the saliva of both piercing-sucking and chewing herbivores (Hogenhout and Bos 2011). Current data demonstrate that specific pest feeding triggers a specific complex signalling pathway in the plant, mainly mediated by hormones, leading to the development of direct defences and general stress-related responses.

Morphological barriers such as waxes or pectins and biosynthesis of gramine, acontic phenolics and amino acids as induced compounds with protective roles have been detected in barley cultivars damaged by aphids (Corcuera 1993; Larsson et al. 2011). Callose deposits have also been observed in longitudinal veins of barley 24 h after *D. noxia* feeding, whereas only high densities of *R. padi* caused weak callose depositions within 7 days of infestation (Saheed et al. 2009). The accumulation of callose has been related to the strong symptoms induced by the Russian wheat aphid (*D. noxia*), and, probably, the presence of callose tries to avoid the reduction of the phloem transport rate in the damaged sieve elements. In contrast, the bird cherry-oat aphid (*R. padi*) did not cause visible symptoms and induced very limited callose deposition (Saheed et al. 2007, 2009). Barley plants are also able to accumulate inhibitors of serine (chymotrypsin and trypsin) and cysteine proteases (cathepsin B-, L- and F-like) and certain PR-proteins (thaumatin, chitinases, β-glucanases) as defence molecules after aphid and acari infestations (Forslund et al. 2000; Casaretto et al. 2004; Cambra et al. unpublished results). In fact, different susceptibility against *S. graminum* and *R. padi* aphids observed in barley cultivars has been correlated to their ability to produce protease inhibitors (Casaretto and Corcuera 1998). Similarly, there have been reported barley resistant lines to *R. padi*-induced chitinase expression in leaves 2 days after aphid treatment, while chitinase levels were only detected after day-7 infection in susceptible lines (Forslund et al. 2000). As described by Smith and Boyko (2007), chemical defences deployed by plants after pest infection may directly self-damage plant tissues. To avoid the autotoxicity, plants induce the expression of detoxification genes such as the high levels of peroxidase and other reactive oxygen species (ROS)-scavenging enzymes, produced in barley as well as in other cereal species after *S. graminum* and *R. padi* feeding (Chaman et al. 2001).

Secondary compounds are also considered to be tightly associated with defence against herbivores, pathogens and/or abiotic stresses. Ninkovic and Ahman (2009) found seven *Hordeum* genotypes significantly affected in the *R. padi* aphid acceptance, among the 19 genotypes treated with volatiles from an undamaged barley cultivar. Moreover, Petterson et al. (1996) showed that volatiles from a barley plant attacked by aphids could induce a change in neighbouring un-attacked plants, making them less acceptable to *R. padi*. These effects were positively correlated with aphid growth indicating that aphids perceived changes in plants as induced by volatiles and suggesting the volatile interaction as a putative component of induced resistance. Recently, volatile organic compounds such as linalool, linalool oxide, α-pinene, hexenol and β-caryophyllene have been detected in significant amounts in barley leaves after *Oulema melanopus* feeding (Piesik et al. 2011). It was previously shown that linalools deter oviposition and attract natural enemies and β-caryophyllene draw parasitoids (Kessler and Baldwin 2001). Volatiles emitted

by plants attacked by herbivores can activate defence mechanisms in neighbouring or even in the same plants making them less suitable to herbivores. Additionally, there have been described multi-trophic interactions among volatiles from different cultivars, termed allelobiosis, with a reduced acceptance of barley plants by *R. padi* (Kellner et al. 2010). The defence function for these volatiles suggests the enormous potential of the manipulation of volatile emission in plants in relationship to pest management in agricultural contexts.

Other important group of small molecules involved in plant defence against pathogens and herbivores are phytohormones. Salicylic acid (SA), jasmonic acid (JA) and ethylene have been described as primary signals of the defence pathway, but more recently, auxins, abscisic acid, gibberellins, cytokinins and brassinosteroids have emerged as key players in the plant immune response. Generally, chemical and volatile organic compounds induced by wounding and chewing insects and necrotrophic pathogens seem to be mainly regulated by JA, while SA coordinates the plant responses after infection of biotrophic pathogens and sucking insects. However, plenty of exceptions have been reported suggesting that the orchestration of plant immune responses requires the interconnexion of a complex signalling network (Pieterse et al. 2000; Robert-Seilaniantz et al. 2011). For example, in barley ethylene is involved in the oxidative and defensive responses to the aphids *S. graminum* and *R. padi*, and its production increases with the degree of aphid infestation (Argandoña et al. 2001). Moreover, an accumulation of SA has also been detected in two barley varieties, UNA-80 and LM-109, in response to *S. graminum* attack (Chaman et al. 2003). Conversely, ABA and GA were earlier described as having no effects on barley feeding of specific species or even specific races of aphids. These regulators applied to healthy leaves and failed to produce the characteristic *D. noxia*-induced damage symptoms of rolling, and the application of these hormones to plants infected by *D. noxia* had no effect on the aphid development (Miller et al. 1994).

12.4 Novel Molecular Approaches to Analyse Plant–Pest Interactions in Barley

Currently, molecular experimental approaches allow the identification of candidate genes/proteins involved in resistance by the comparison of differential responses between susceptible and tolerant genotypes after pest feeding. Changes in plant responses induced by pests have been mainly analysed using transcriptional profiling in a high number of plant–pest interactions (Soria-Guerra et al. 2010; Smith et al. 2010; Zhou et al. 2011). More recently, comparative proteomics and metabolomics are being used to investigate the molecular interactions between plants and pests (Collins et al. 2010; Ferry et al. 2011; Liu et al. 2010; Maserti et al. 2011).

As far as we know, little is known about barley gene expression profiles in response to pests. Only two studies (Delp et al. 2009; Gutsche et al. 2009) have performed microarray analysis of barley plants to identify candidate resistance-related genes, and both of them have selected aphids as a target pest of the study. The natural existence of the barley genotypes partially tolerant or susceptible to aphids offers an excellent strategy to design microarray experiments in both reports. In response to the Russian wheat aphid feeding (*D. noxia*), a total of 909 genes showed significant differences in their expression levels in the tolerant barley as compared to susceptible plants. Among them, two peroxidase genes up-regulated to a greater degree in tolerant genotypes were deeply analysed. Based on the results, the authors hypothesised that the high level of peroxidases could help to efficiently remove ROS accumulated in response to aphid feeding in the tolerant barley plants (Gutsche et al. 2009). However, experiments of over-expressing and down-regulating targeted genes need to be done to provide direct insights into the tolerant responses.

Delp et al. (2009) performed a complex selection of genotypes to carry out transcriptomic assays in order to verify differences between susceptible and tolerant plants to *R. padi*. They used two barley cultivars, Lina and Kara, both susceptible to *R. padi*, one resistant wild barley accession (*H. vulgare*. ssp. *spontaneum* 5, Hsp5) and one resistant doubled haploid breeding line (DH28:4) derived from a cross between Hsp5 and Lina and, subsequently, a backcross to Lina. After the bird cherry-oat aphid (*R. padi*) infestation, large differences in gene expression induction between the two susceptible versus the two tolerant barley genotypes were found. The most expressed genes in resistant lines encoded a calcium-binding protein, a proteinase inhibitor, a methyl jasmonate-inducible lipoxygenase, a putative Ser/Thr kinase and several thionins. These genes were validated by qRT-PCR and selected as candidate genes with putative resistant properties to be used for defence against phloem-feeding aphids (Delp et al. 2009). Further studies using breeding populations, wider defence characterisation and QTL analysis related to resistance phenotype need to be developed to complete the transcriptomic analysis.

Besides barley, the microarray technology has been used in other cereals to analyse interaction with the Russian wheat aphid in wheat (Botha et al. 2006; Boyko et al. 2006; Smith et al. 2010) and the greenbug in sorghum (Zhu-Salzman et al. 2004; Park et al. 2006). Many of the differentially expressed genes encoded proteins potentially involved in defence, but genes dealing with the cell wall synthesis, photosynthetic processes, oxidative stress and primary and secondary metabolism have also been identified. Most transcriptional changes in response to pest attack were evident and could be correlated to differential visible phenotype plant symptoms specifically caused by aphid species. Presently, only one study has analysed changes in the proteome of a cereal after 24 h of aphid feeding. Sixty-seven protein spots differed significantly between control and infested wheat plants with *S. avenae*, and most of them were involved in the same plant physiological processes as stated above (Ferry et al. 2011). However, further studies are required to confirm and identify differentially expressed proteins.

12.5 Barley as Source of Putative Insecticidal Transgenes

Traditionally, pest control has based on the combination of insecticides/acaricides, host plant resistance and biological control. The natural plant variation related to defence mechanisms against pests has resulted from their co-evolution, and eventually, it has facilitated the identification of effective sources of resistance. However, this approach has been poorly exploited in agriculture (Broekgaarden et al. 2011). Alternatively, biotechnological approaches have allowed the stable expression of insecticidal transgenes into crops to enhance tolerance against pests, reducing the use of pesticides. The potential of Bt toxins is well known, first used as topical pesticides to protect crops and more recently expressed in transgenic plants to confer inherent pest resistance (Sanahuja et al. 2011). Transgenic Bt barley has not been created, and it can be considered an exception within the more than 40 Bt-crop species already generated. In contrast, there are many examples in which barley genes with anti-insect and anti-mite properties have been transferred into the plant genome of mono- and dicotyledonous plants to enhance pest resistance.

The wider group of barley genes used as defence transgenes against pests corresponds to protease inhibitor (PI) families, in particular inhibitors of serine and cysteine proteases. The defence role of PIs is based on their ability to block the major proteolytic digestive enzymes from herbivorous arthropods and subsequently to reduce the insect/mite viability, development and performance. Altpeter et al. (1999) and Alfonso-Rubi et al. (2003) produced transgenic wheat and rice lines, respectively, expressing the trypsin inhibitor CMe from barley. Wheat and rice transgenic seeds conferred a significant reduction of the survival rate of two important storage pests, the lepidopteran *Sitotroga cerealella* and the coleopteran *Sitophilus oryzae*. In parallel, a decrease in the trypsin-like activity of insect crude midgut confirmed the utility of the barley transgene to inhibit the digestive process of these cereal grain pests. Conversely, larvae of *Spodoptera exigua* were able to adapt their digestive physiology to barley CMe gene transgenically expressed in tobacco under the 35S CaMV promoter. The 25 % reduction in the trypsin-like activity observed in larval midgut after being reared with CMe tobacco leaves was compensated with a significant induction in other protease activities (Lara et al. 1999). To avoid the insect adaptation, the pyramiding approach that combines genes encoding PIs with other resistance genes has been developed as a method to prevent pest resistance acquisition and to improve pest control. Thus, significant increase in mortality and strong negative effects on development of the *Helicoverpa armigera* larvae were observed after feeding on transgenic tobacco leaves expressing both a PI from *N. alata* and the β-hordeothionin from *H. vulgare,* in comparison with tobacco plants containing either gene alone (Charity et al. 2005).

Regarding the barley cysteine-protease inhibitors, known as cystatins, Alvarez-Alfageme et al. (2007) showed the negative effects of potato plants expressing a variant of the barley cystatin HvCPI-1 on the growth and digestive physiology of the Colorado potato beetle (*Leptinotarsa decemlineata*). Additionally, the prey-

mediated effects of the barley cystatin were analysed at the third trophic level, using the spined soldier bug (*Podisus maculiventris*). No effects on survival and growth were observed when *P. maculiventris* nymphs were exposed to barley cystatin by predation on Colorado potato beetle larvae reared on transgenic potato plants. This study clearly demonstrated that the expression of the barley cystatin variant in potato did not represent a risk to the useful natural enemy *P. maculiventris*. More recently, the potential of the barley cystatin HvCPI-6 as pest control protein has been successfully shown by feeding experiments impaired on transgenic lines of *Arabidopsis* and maize expressing this encoding cystatin gene. The barley inhibitor reduced the performance of two aphid species in artificial and transgenic *Arabidopsis* plants (Carrillo et al. 2011a). Similarly, the development and reproductive performance of the spider mite *Tetranychus urticae* was negatively affected when acari larvae were fed with maize plants expressing the HvCPI-6 cystatin (Carrillo et al. 2011b).

To avoid the insect adaptation, the pyramiding approach that combines genes encoding different PIs or PIs with other resistance genes has been developed as a method to prevent pest resistance acquisition and to improve pest control. Thus, significant increase in mortality and strong negative effects on development of the *Helicoverpa armigera* larvae were observed after feeding on transgenic tobacco leaves expressing both a PI from *N. alata* and the β-hordeothionin from *H. vulgare,* in comparison with tobacco plants containing either gene alone (Charity et al. 2005).

Recently, Santamaria et al. (2012) have reported the potential of pyramiding two classes of plant protease inhibitors (CMe and Hv-CPI6) to prevent plant damage caused by the two-spotted spider mite, *Tetranychus urticae*.

Besides examples of barley genes already used for pest control, there are other genes isolated from *Hordeum* species integrated into plant genomes to confer other beneficial traits which could also be used to enhance pest resistance. Among them, it is important to mention that the barley class II chitinase gene transgenically expressed in wheat is able to reduce the *Fusarium graminearum* infection (Shin et al. 2008). Anti-mite properties of chitinase genes from distinct origin have already been reported (McCafferty et al. 2006). Therefore, transgenic plants expressing defence genes targeted to silence or inhibit essential pathogen processes are promising alternatives to control arthropod pest.

Conclusions

Taken these data altogether, and although damage from field phytophagous arthropods is not a major threat for barley, the barley–pest interaction has been deeply studied to understand plant responses and to fight towards herbivore attack, particularly against aphids. Besides structural barriers differentially developed by *Hordeum* genotypes in response to pest infestation, protective molecules including secondary metabolites, mainly volatiles, are

(continued)

induced. Volatiles may also function as signals to prime defence responses of neighbouring barley plants not still infected. In addition, phytohormones modulate barley pathways to elicit anti-herbivore defences.

Finally and based on the high basal resistance of barley to be attacked by insects and mites, it has been used as a source of transgenes with insecticidal/acaricidal properties. The integration of candidate barley genes with defence functions into plant genomes may generate resistance against pests. These biotechnological approaches are a promising alternative to control arthropod pests to be included in conventional plant-assessment programmes in a foreseeable future.

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References

Ahman I, Tuvesson S, Johasson M (2000) Does indole alkaloid gramine confer resistance in barley to the aphid *Rhopalosiphum padi*? J Chem Ecol 26:233–255

Alfonso-Rubi J, Ortego F, Castañera P, Carbonero P, Diaz I (2003) Transgenic expression of trypsin inhibitor CMe from barley in indica and japonica rice, confers resistance to the weevil *Sitophilus oryzae*. Transgenic Res 12:23–31

Altpeter F, Diaz I, McAuslane H, Gaddour K, Carbonero P, Vasil IK (1999) Increased insect resistance in transgenic wheat stably expressing trypsin inhibitor CMe. Mol Breed 5:53–63

Alvarez-Alfageme F, Martinez M, Pascual-Ruiz S, Castañera P, Diaz I, Ortego F (2007) Effects of potato plants expressing a barley cystatin on the predatory bug *Podisus maculiventris* via herbivorous prey feeding on the plant. Transgenic Res 16:1–13

Argandoña VH, Chaman M, Cardemil L, Muñoz O, Zuñiga GE, Coprcuera LJ (2001) Ethylene production and peroxidase activity in aphid-infested barley. J Chem Ecol 27:53–67

Botha AM, Lacock L, van Niekerk C, Matsioloko MT, du Preez FB, Loots S, Venter E, Kunert KJ, Cullis CA (2006) Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. "TugelaDN" a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)? Plant Cell Rep 25:41–54

Boyko EV, Smith CM, Thara VK, Bruno JM, Deng Y, Starley SR, Klaahsen DL (2006) Molecular basis of plant gene expression during aphid invasion: wheat Pto- and Pts-like sequences are involved in interactions between wheat and Russian wheat aphid (Homoptera: Aphididae). J Econ Entomol 99:1430–1445

Broekgaarden C, Snoeren TAL, Dicke M, Vosman B (2011) Exploiting natural variation to identify insect-resistance genes. Plant Biotechnol J 9:819–825

Carrillo L, Martinez M, Ramessar K, Cambra I, Castañera P, Ortego F, Diaz I (2011a) Expression of a barley cystatin gene in maize enhances resistance against phytophagous mites by altering their cysteine-proteases. Plant Cell Rep 30:101–112

Carrillo L, Martinez M, Alvarez-Alfageme F, Castañera P, Smagghe G, Diaz I, Ortego F (2011b) A barley cysteine-proteinase inhibitor reduces the performance of two aphid species in artificial diets and transgenic Arabidopsis plants. Transgenic Res 20:305–319

Casaretto JA, Corcuera LJ (1998) Proteinase inhibitor accumulation in aphid infested barley leaves. Phytochemistry 49:2279–2286
Casaretto JA, Zuñiga GE, Corcuera LJ (2004) Abscisic acid and jasmonic acid affect proteinase inhibitor activities in barley leaves. J Plant Physiol 161:389–396
Chaman ME, Corcuera LJ, Zúñiga GE, Cardemil L, Argandoña VH (2001) Induction of soluble and cell wall peroxidases by aphid infestation in barley. J Agric Food Chem 49:2249–2253
Chaman ME, Copaja SV, Argandona VH (2003) Relationships between salicylic acid content, phenylalanine ammonia-lyase (PAL) activity, and resistance of barley to aphid infestation. J Agric Food Chem 51:2227–2231
Charity JA, Hughes P, Anderson MA, Bittisnich DJ, Whitecross M, Higgins TJV (2005) Pest and disease protection conferred by expression of barley β–hordothionin and *Nicotiana alata* proteinase inhibitor genes in transgenic tobacco. Funct Plant Biol 32:35–44
Collins RM, Afzal M, Ward DA, Prescott MC, Sait SM, Rees HH, Tomsett AB (2010) Differential proteomic analysis of *Arabidopsis thaliana* genotypes exhibiting resistance or susceptibility to the insect herbivore, *Plutella xylostella*. PLoS One 5:210103
Corcuera LJ (1993) Biochemical basis for the resistance of barley to aphids. Phytochemistry 33:741–747
Davis JA, Radcliffe EB (2008) Reproduction and feeding behaviour of *Myzus persicae* on four cereals. J Econ Entomol 101:9–16
Delp G, Gradin T, Åhman I, Jonsson LMV (2009) Microarray analysis of the interaction between the aphid *Rhopalosiphum padi* and host plants reveals both differences and similarities between susceptible and partially resistant barley lines. Mol Genet Genomics 281:233–248
Ferry N, Stavroulakis S, Guan W, Davison GM, Bell HA, Weaver RJ, Down RE, Gatehouse JA, Gatehouse AMR (2011) Molecular interactions between wheat and cereal aphid (*Sitobion avenae*): analysis of changes to the wheat proteome. Proteomics 11:1985–2002
Forslund K, Pettersson J, Bryngelsson T, Jonsson L (2000) Aphid infestation induces PR-proteins differentially in barley susceptible or resistant to the bird cherry-oat aphid (*Rhopalosiphum padi*). Physiol Plant 110:496–502
Gardenhire JH (1979) Breeding for greenbug *Schizaphis graminum* (Rodani) resistance in wheat and in other small grains. In: Harris MK (ed) Biology and breeding for resistance to arthropods and pathogens in agricultural plants. A&M University, College Station, TX, pp 237–244
Gutsche A, Heng-Moss T, Sarath G, Twigg P, Xia Y, Lu G, Mornhinweg D (2009) Gene expression profiling of tolerant barley in response to *Diuraphis noxia* (Hemiptera: Aphididae) feeding. Bull Entomol Res 99:163–173
Hogenhout SA, Bos JIB (2011) Effector proteins that modulate plant-insect interactions. Curr Opin Plant Biol 14:422–428
Kellner M, Brantestam AK, Ahman I, Ninkovic V (2010) Plant volatile-induced aphid resistance in barley cultivars is related to cultivar age. Theor Appl Genet 121:1133–1139
Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatiles emissions in nature. Science 291:2141–2144
Lara P, Ortego F, Gonzalez-Hidalgo E, Castañera P, Carbonero P, Diaz I (1999) Adaptation of *Spodoptera exigua* (Lepidoptera: Noctuidae) to barley trypsin inhibitor BTI-CMe expressed in transgenic tobacco. Transgenic Res 9:169–178
Larsson KAE, Saheed SA, Gradin T, Delp G, Karpinska B, Botha CEJ, Jonsson LMV (2011) Differential regulation of 3-aminomethylindole/N-methyl-3-aminomethylindole N-methyltransferase and gramine in barley by both biotic and abiotic stress conditions. Plant Physiol Biochem 49:96–102
Liu C, Hao F, Hu J, Zhang W, Wan L, Zhu L, Tang H, He G (2010) Revealing different systems responses to brown plant hopper infestation for pest susceptible and resistant rice plants with the combined metabolomic and gene-expression analysis. J Proteome Res 9:6774–6785
Maserti BE, Carratore R, Della Croce CM, Podda A, Migheli Q, Froelicher Y, Luro F, Morillon R, Ollitrault P, Talon M, Rossignol M (2011) Comparative analysis of proteome changes induced

by the two spotted spider mite *Tetranychus urticae* and methyl jasmonate in citrus leaves. J Plant Physiol 168:392–402
McCafferty HRK, Moore PH, Zhu Y (2006) Improved *Carica papaya* tolerance to carmine spider mite by the expression of *Manduca sexta* chitinase transgene. Transgenic Res 15:337–347
Miller HL, Neese PA, Ketring DL, Dillwith JW (1994) Involvement of ethylene in aphid infestation of barley. J Plant Growth Regul 13:167–171
Mozos-Pascual M (1997) Plagas de los productos almacenados. Bol SEA 20:93–119
Ninkovic V, Ahman I (2009) Aphid acceptance of *Hordeum* genotypes is affected by volatile exposure and is correlated with aphid growth. Euphytica 169:177–185
Park SJ, Huang Y, Ayoubi P (2006) Identification of expression profiles of sorghum genes in response to greenbug phloem-feeding using cDNA subtraction and microarray analysis. Planta 223:932–947
Petterson J, Quiroz A, Fahad AE (1996) Aphid antixenosis mediated by volatiles in cereals. Acta Agric Scand Sect B Soil Plant Sci 46:135–140
Piesik D, Panka D, Delaney KJ, Skoczeck A, Lamparski R, Weaver DK (2011) Cereal crop volatile organic compound induction after mechanical injury, beetle herbivory (Oulema spp.) or fungal infection (Fusarium spp.). J Plant Physiol 168:878–886
Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2000) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308–316
Porter DR, Mornhinweg DW, Webster JA (1999) Insect resistance in barley germplasm. In: Clemet SL, Quinsenberry SS (eds) Global plant genetic resources for insect-resistant crops. CRC Press LLC, Boca Raton, FL, pp 51–61
Robert-Seilaniantz A, Grant M, Jones JDG (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonisms. Annu Rev Phytopathol 49:317–343
Saheed SA, Botha CEJ, Liu L, Jonsson L (2007) Comparison of structural damage caused by Russian wheat aphid (*Diuraphis noxia*) and Bird cherry-oat aphid (*Rhopalosiphum padi*) in a susceptible barley cultivar *Hordeum vulgare* cv. Clipper. Physiol Plant 129:429–435
Saheed SA, Cierlik I, Larsson KAE, Delp G, Bradley G, Jonsson L, Botha CEJ (2009) Stronger induction of callose deposition in barley by Russian wheat aphid than bird cherry-oat aphid is not associated with differences in callose synthase or beta-1,3-glucanase transcript abundance. Physiol Plant 135:150–161
Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol J 9:283–300
Santamaria ME, Cambra I, Martinez M, Pozancos C, Gonzalez-Melendi P, Gbric V, Castañera P, Ortego F, Diaz I (2012) Gene pyramiding of peptidase inhibitors enhances plant resistance to the spider mite *Tetranychus urticae*. PLoS One 7:e43011
Shin S, Mackintosh CA, Lewis J, Heinen SJ, Radmer L, Dill-Macky R, Baldridge GD, Zeyen RJ, Muehlbauer GJ (2008) Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*. J Exp Bot 59:2371–2378
Shufran KA (2011) Host race evolution in *Schizaphis graminum* (Hemiptera: Aphididae): nuclear DNA sequences. Environ Entomol 40:1317–1322
Smith CM, Boyko EV (2007) The molecular bases of plant resistance and defense responses to aphid feeding: current status. Entomol Exp Appl 122:1–16
Smith CM, Liu X, Wang LJ, Liu X, Chen M-S, Starkey S, Bai J (2010) Aphid feeding activates expression of a transcriptome of oxylipin-based defense signals in wheat involved in resistance to herbivory. J Chem Ecol 36:260–276
Soria-Guerra RE, Rosales-Mendoza S, Chang S, Haudenshield JS, Padmanaban A, Rodriguez-Zas S, Hartman GL, Ghabrial SA, Korban SS (2010) Transcriptome analysis *of* resistant and susceptible genotypes of *Glycine tomentella* during *Phakopsora pachyrhizi* infection reveals novel rust resistance genes. Theor Appl Genet 120:1315–1333
Webster JA, Baker CA, Porter DR (1991) Detection and mechanism of Russian wheat aphid (Homoptera: Aphididae) resistance in barley. J Econ Entomol 84:669–673

Weng Y, Perumal A, Burd JD, Rudd JC (2010) Biotypic diversity in greenbug (Hemiptera: Aphididae): microsatellite-based regional divergence and host-adapted differentiation. J Econ Entomol 103:1454–1463

Zhou G, Wang X, Yan F, Wang X, Li R, Cheng J, Lou Y (2011) Genome-wide transcriptional changes and defence-related chemical profiling of rice in responses to infestation by the rice striped stem borer *Chilo suppressalis*. Physiol Plant 143:21–40

Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H (2004) Transcriptional regulation of sorghum defense determinants against a phloem-feeding aphid. Plant Physiol 134:420–431

Chapter 13
Molecular Farming

Einar Mäntylä and Björn L. Örvar

13.1 Introduction

The manufacturing of recombinant proteins in plants has emerged as a viable alternative to bacterial, fungal or animal cell-based expression systems. The use of plants for the production of such novel valuable compounds is commonly defined as molecular farming. Molecular farming presents a sustainable, green manufacturing technology for high value products of biological origin and a promising innovative hi-tech sector in agriculture and horticulture.

A number of plant species have been tested throughout the years as host organisms, both dicots and monocots and domesticated and less domesticated plants with both stable transgenic and transient expression approaches. The host tissue accumulating the recombinant proteins has varied from secretion through roots to accumulation in tubers, leafs, flowers, fruits and seeds (Yano et al. 2010; Sharma and Sharma 2009; Basaran and Rodríguez-Cerezo 2008; Stoger et al. 2005; Drake et al. 2003). Not only have the different tissues been targeted for protein accumulation, but intracellular localisation has been explored as well, such as retention in the endoplasmic reticulum; accumulation in organelles such as chloroplasts, protein bodies and cytosol and secretion out of the cell (Streatfield 2007; Kamenarova et al. 2005; Ma et al. 2005). The optimal path for the protein and its final cellular destination may vary depending on the protein, the plant species and the tissue it is accumulated in. The biological diversity of plants and their

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eucaryotic nature at the cellular level offer many strategies for the manufacturing of recombinant proteins, and different strategies have their strengths and weaknesses. The accumulation of recombinant proteins can be tissue specific or systemic in the whole plant, depending on the nature of the promoters chosen to drive gene expression. Plant cells grown in suspension culture can and have been used successfully for the production of recombinant proteins. In fact, the first plant-made pharmaceutical to be approved by the FDA is a glycosylated enzyme, glucocerebrosidase, produced by suspension culture of carrot cells (Protalix Inc.). Suspension culture of plant cells can be considered as a hybrid manufacturing technology between traditional bioreactors and whole plant approaches.

Considering whole plants, amongst the strategies to choose from are whether to harvest the protein from rapidly growing, highly metabolically active green tissue or metabolically quiescent storage tissue. Target peptides added to the protein can be used to direct the protein accumulation to various intracellular compartments that may increase stability or affect the level and nature of post-translational modification of the recombinant product. The choice of a strategy will affect protein yield, the protein environment in the host plant and the extract, the downstream process and ultimately the economics and feasibility of the molecular farming and may even affect the quality of the product.

The interest in developing molecular farming is partly based on the ample existing know-how and technology adapted to domesticated plants; the agricultural infrastructure for any scale of cultivation and harvesting is present for any crop species, and in many cases, suitable postharvest treatments such as seed processing and storage for seed-based systems are known. To put it simply, the large-scale upstream processes were honed to perfection by agricultural practices before the term of molecular farming was even phrased. Existing infrastructure with solar energy driving photosynthesis and sustainable growth of the biomass provide an attractive economical and environmental incentive to develop molecular farming into a green, high-value low-carbon footprint industry, with a potential to contribute significantly to the bioeconomy of the future.

Cereals have been intensively bred to produce large harvestable grains, accumulating polysaccharides, lipids and specific storage proteins in the stable, metabolically quiescent tissue of grains. Grains stored under proper conditions can last dormant for years, maintaining the proteins intact for an extended period of time.

The major commercially important cereals, maize, rice, wheat and barley have all been genetically transformed (Ramessar et al. 2008a, b; Fujiwara et al. 2010; Han et al. 2012; Erlendsson et al. 2010; Hensel 2011), representing major crops in both temperate (wheat and barley) and warmer regions (maize and rice). Recombinant proteins have been demonstrated to accumulate at high levels in grains of cereals (Christou et al. 2008; Boothe et al. 2010).

Molecular farming in seed-based systems is considered promising for vaccine production, and recombinant antigens produced in seeds have been demonstrated to remain stable and immunogenic in both animals and humans for over 18 months when stored without refrigeration, thus eliminating the necessity of costly cold

chain for vaccine delivery in the developing world (Penney et al. 2011; Nochi et al. 2007).

Barley has a number of qualities that make it an excellent platform for the manufacturing of recombinant proteins with molecular farming. The combination of genetics, physiology, agricultural suitability, inherent containment properties, infrastructure and long history of domestication of barley contribute to the benefits of barley for industrial cultivation. This review attempts to describe some of the developments of sustainable molecular farming industry based on grain-based molecular farming with barley.

13.2 Benefits of Barley for Molecular Farming

When considering a host organism for the production of technical proteins, or proteins of medical relevance, i.e. protein products for non-food, non-feed purposes, a number of challenges and criteria need to be considered, such as toxicity, safety, agricultural practices, containment strategies, the applicability of molecular biology and tissue culture and biochemistry and downstream processing.

The use of crop plants for molecular farming provides obvious benefits in terms of their long history of agricultural and ecological interactions with the environment, safety and efficient management under different conditions. Well-defined varieties through long and extensive breeding provide farmers with stable crop plants with a well-synchronised life cycle, manageable cultivation and efficient harvesting. Domestication is a process of increasing interdependence of humans and target plant or animal populations (Zeder et al. 2006). Domesticated crop species furnish with a long history of safety of the crop compared to poorly domesticated plants with high variation in life cycle, possible invasiveness and largely unknown potentially toxic metabolic responses to abiotic and biotic stresses. Furthermore, the reduced competitiveness and invasiveness of crop plants under noncultivated conditions and general dependency to human intervention to successfully complete their life cycle contribute to the safe use of crop plants from the point of environmental concerns.

Barley certainly is a crop plant with a long history of domestication reaching back thousands of years and has been adapted and bred to emphasise yield and other agricultural traits to accommodate the needs of humans for mainly feed and brewing purposes (see also Chaps. 1 and 3).

The relatively long life cycle, self-pollination, large, few and heavy grains that are poorly suited to wind dispersal may contribute to poor invasiveness and efficient confinement of the annual plant barley. Another benefit of barley is the possibility to obtain doubled haploids, which is useful to improve yields in molecular farming and stability of elite production lines (see also Chap. 20). In addition, barley is an annual plant that does not persist in natural habitats or fields without intervention, making cultivation of barley for molecular farming a reversible and highly

manageable operation. Molecular farming with barley can be ceased (like any other barley cultivation) with no lasting effect on the environment, positive or negative.

Production of recombinant proteins in grains of barley is, however, a time-consuming strategy, compared to cell suspension culture or transient expression, as stably transformed elite product lines need to be generated, defined and propagated before downstream processing and production can commence.

The fact that barley has a long history of domestication implies that it is a food but mainly a fodder plant. It can be argued that molecular farming should be restricted to non-food, non-fodder plants to avoid the risk of contamination of the food chain with material not intended for consumption. While this may seem a reasonable demand, it needs to be weighed against the alternative, conducting molecular farming in non-domesticated plants with limited knowledge available on the genetics, biochemistry, biology and ecological behaviour of a non-domesticated plant that by definition would not be dependent on human intervention. The risk of unforeseen events at every level is greater than with a plant with known agronomic traits and established procedures.

13.3 Inherent Containment Properties

Amongst the valuable biological containment features of barley is the high level of self-pollination that effectively bars cross-fertilisation between adjacent plants (Ritala et al. 2002; Nair et al. 2010). An indication of the containment is the short isolation distances required to maintain purity of barley cultivars. Canadian Seed Growers' Association stipulates isolation distances for certification of foundation, registered or certified non-hybrid barley seed from different varieties of barley to be 3 m, indicating the ease of maintaining barley varieties isolated and the low risk of gene flow (Canadian Seed Growers' Association 2013). Several varieties are characterised by almost complete self-pollination such as the Golden Promise variety that is preferentially used for genetic transformation, including molecular farming (Hensel et al. 2008; Erlendsson et al. 2010). Self-pollination (cleistogamy) of barley occurs where pollination has taken place before the flower opens and before the pollen can be dispersed from the flower. Thus, when the pollen is released, all flowers that are open are already pollinated and not receptive to external pollen, effectively abolishing the possibility of cross-pollination between barley plants. The underlying genetic and molecular mechanism behind cleistogamy in barley cultivars has recently been elucidated (Nair et al. 2010).

Cross-pollination of barley under field conditions has been studied by Ritala et al. (2002) and more recently by Hermannsson et al. (2010), who conducted a multiyear experiment in a subarctic climate where Golden Promise was planted in 20–30 cm distance from another developmentally compatible six-rowed variety. Hybrid offspring have a distinct phenotype, as established by forced cross-pollination. This allows fast screening of a large population of offspring for rare cross-pollination events between barley varieties grown immediately adjacent to

each other. The experiment by Hermannsson and colleagues confirmed the very low frequency of cross-pollination between Golden Promise and the six-rowed cultivar Ven. In the larger setup of the experiment, not a single hybrid was detected in the 600,000 plants screened, despite the short distance between the compatible cultivars. The results show that maintaining moderate distance from other barley is a safe and simple measure to efficiently prevent gene flow between barley crops.

Lifetime of barley pollen in general is very short, and barley pollen is not distributed by insects (USDA 2006). The viability of the pollen released from the Golden Promise variety was studied by researchers at the MALTAgen Forschung Company indicating that the pollen was already non-viable by the time it was released from the flower (oral communication—MALTAgen Forschung). This reproductive behaviour of barley varieties like Golden Promise is important from the point of molecular farming, as it provides an extraordinary level of contained cultivation, adding to the safety, and facilitates quality control of molecular farming with barley whether in field or in greenhouse conditions.

Barley grains are heavy, on average 30 mg/grain, greatly limiting wind dispersal of barley grains. An opportunity to measure the level of wind dispersal of grain under extreme conditions rose after a severe storm hit a fully developed Golden Promise barley plot ready to be harvested. The storm speed measured 44 m/s, and grain dispersal down the wind direction was measured to be maximum 35 m from the nearest plants. According to seed counts, 99.9 % of the seeds shed by the storm were within 5 m radius from the plants despite the heavy storm (ORF Genetics, unpublished results).

This further corroborates the inherent containment properties and manageability of barley even under extreme field conditions. Barley stays on the field.

The superb biological containment exhibited by barley has not only implications for responsible contained molecular farming; it has relevance for the economy of molecular farming operations as well, both for in field and greenhouse cultivation of barley. High-density cultivation of barley lines becomes possible without the risk of contamination between lines, as the need for isolation distance is minimal, enabling efficient use of land and greenhouse space. Under greenhouse conditions, the containment attributes of barley become especially valuable; transgenic barley lines producing different proteins can be cultivated in the same greenhouse without the risk of cross-contamination. In fact, during 8 consecutive years of all-year-round greenhouse cultivation of transgenic Golden Promise lines, no examples of cross-fertilisation between thousands of GP lines have been observed despite high-density cultivation (20 cm distance between different GP lines) (ORF Genetics, unpublished results). Subsequently, greenhouse cultivation of multiple lines becomes more economical and amenable to high level of automation, and greenhouse facilities become effectively multiproduct facilities. Molecular farming with barley in hydroponic culture on conveyor belts has proven successful and economical for the production of a variety of small to moderate volumes of recombinant proteins (Fig. 13.1). The feasibility of scaleup in greenhouse or field conditions for any given product of molecular farming depends largely on the combination of the

Fig. 13.1 Molecular farming with barley in geothermally heated greenhouses near Reykjavik, Iceland, courtesy of ORF Genetics

type of recombinant protein, its intended application, and the economics and market properties of the protein.

Heavy regulatory burden associated with European regulations on GMOs that are designed to have strict control on widespread agricultural use of genetically engineered plants for food and fodder production across Europe is poorly suited to address the manageable, localised and limited scope of cultivation required by molecular farming of valuable compounds for other uses. Such poorly developed regulatory environment stifles the advancement of responsible field-based molecular farming and the R&D activities by universities, research institutes and SMEs, effectively handing over a monopoly to large multinationals that have the resources to tackle the costly and time-consuming regulatory process.

To develop further the safety aspects of molecular farming and to address concerns of possible postharvest mixing of molecular farming crops with other

Fig. 13.2 Heads of Dimma cultivar to the *left* and of the Golden Promise cultivar to the *right*

crops, ORF Genetics, in collaboration with the Icelandic Agricultural University, has set out to develop the new barley variety "Dimma" that is self-pollinating and both amenable to transformation and tissue culture but is black in colour. The resulting barley grains are easily recognisable from ordinary barley to the resolution of single grains. This makes postharvest monitoring possible and provides a tool to effectively prevent postharvest mixing of molecular farming with barley harvested for other purposes (Fig. 13.2).

13.4 Grains and Encapsulated Proteins

Grains as target tissue for recombinant protein accumulation have several advantages over protein accumulation in metabolically active green tissue: harvestability, compactness, encapsulation of the recombinant product, stability of proteins within the grains, long-term storage at ambient temperatures, low bioburden in the grain, relatively simple protein profile, scaleable processing, existing infrastructure and initial processing and operational flexibility by separation of upstream (cultivation) and downstream (protein purification) operations. The use of seeds in general for molecular farming has been discussed in a review (Boothe et al. 2010). The attenuating metabolism with reduced protein turnover and dwindling proteolytic activity and the onset of storage protein synthesis provides optimal timing and environment for recombinant protein accumulation. A way to take advantage of this in seed-based molecular farming is to have strong seed-specific promoters from storage protein genes to drive the expression of the recombinant gene. Control of transgene expression in Triticeae cereals has been reviewed recently by Hensel et al. (2011), with detailed listing of various promoter studies on target cereal species addressing both ubiquitous and tissue-specific promoters, promoters responsive to abiotic and biotic stresses and target peptides for intracellular targeting of proteins. Useful grain-specific promoters include barley *B1 hordein* and barley *D hordein* (Cho et al. 2002), barley α-*amylase* (Caspers et al. 2001), oat *globulin* 1 (Vickers et al. 2006) and rice *Glutenin B1* (Patel et al. 2000). Grain-specific expression in barley offers the possibility for controlled tissue-specific expression of a gene and accumulation of the corresponding recombinant protein in a tissue entering the quiescent state of dormancy. The recombinant proteins accumulate subsequently only in a tissue-specific manner during the late stages of the life cycle of the plant. An example of this is the endosperm-specific accumulation of growth factors listed in Table 13.1. With the grain maturation and desiccation comes the benefit of extended storage of the accumulated product under aseptic conditions inside the grain.

Steiner and Ruckenbauer (1995) verified the effectiveness of ultradry storage of cereal grains in a hermetically sealed container at ambient temperature. After 110 years of storage at 10–15 °C and ultradry conditions, barley grains of about 3.1 % moisture exhibited 90 % germination. Although there is no data on the condition of the storage proteins specifically, it must be concluded that genetic, biochemical and cellular condition of the grains must be intact for germination to occur so effortlessly.

The key to such longevity of the grain is the cessation of metabolic activity that follows the maturation and desiccation of the grain, combined with favourable (dry) storage conditions. Low metabolic and proteolytic activity provides a stable environment for any proteins that were accumulated in the seed during maturation, compared to the protein turnover in metabolically active cells. An example of metabolic activity that has relevance to recombinant protein accumulation is the activity of proteases in the host cells. The proteolytic activity of barley grain extract was compared to sonicated extracts of the BL21 strain of *E. coli*, a common strain

Table 13.1 Overview of recombinant proteins produced in barley for molecular farming purposes

Recombinant protein	Full name of protein	a.a. sequence	Protein type	Source
ANG	Angiogenin-1	Human	Growth factor	ORF Genetics
EGF	Epidermal growth factor	Human	Growth factor	ORF Genetics
EMAP-2	Endothelial-monocyte activating polypeptide-II	Human	Growth factor	ORF Genetics
FGF basic	Fibroblast growth factor	Human	Growth factor	ORF Genetics
Flt3-ligand	FMS related tyrosine kinase 3 ligand	Human	Growth factor	ORF Genetics
G-CSF	Granulocyte-colony stimulating factor	Human	Growth factor	ORF Genetics
GDNF	Glial cell line derived neurotrophic factor	Human	Growth factor	ORF Genetics
HB-EGF	Heparin binding EGF	Human	Growth factor	ORF Genetics
IFN alpha 2a	Interferon alpha 2a	Human	Growth factor	ORF Genetics
IFN gamma	Interferon gamma	Human	Growth factor	ORF Genetics
IL-16	Interleukin-16	Human	Growth factor	ORF Genetics
IL-1α	Interleukin-1 alpha	Human	Growth factor	ORF Genetics
IL-2	Interleukin-2	Human	Growth factor	ORF Genetics
IL-3	Interleukin-3	Human	Growth factor	ORF Genetics
IL-4	Interleukin-4	Human	Growth factor	ORF Genetics
IL-5	Interleukin-5	Human	Growth factor	ORF Genetics
IL-6	Interleukin-6	Human	Growth factor	ORF Genetics
IL-7	Interleukin-7	Human	Growth factor	ORF Genetics
IL-9	Interleukin-9	Human	Growth factor	ORF Genetics
IL-21	Interleukin-21	Human	Growth factor	ORF Genetics
KGF	Keratinocyte growth factor	Human	Growth factor	ORF Genetics
LIF	Leukemia inhibitory factor	Human	Growth factor	ORF Genetics

(continued)

Table 13.1 (continued)

Recombinant protein	Full name of protein	a.a. sequence	Protein type	Source
LIF	Leukemia inhibitory factor	Human	Growth factor	ORF Genetics
M-CSF	Macrophage colony stimulating factor	Human	Growth factor	ORF Genetics
NRG1 (HRG-beta-2)	Neuregulin 1	Human	Growth factor	ORF Genetics
PDGF-BB	Platelet-derived growth factor-BB	Human	Growth factor	ORF Genetics
RANKL	Receptor activator of NF-kappaB ligand	Human	Growth factor	ORF Genetics
SCF	Stem cell factor	Human	Growth factor	ORF Genetics
SCF	Stem cell factor	Human	Growth factor	ORF Genetics
SF20	Stromal cell-derived growth factor	Human	Growth factor	ORF Genetics
TNF alpha	Tumor necrosis factor, alpha	Human	Growth factor	ORF Genetics
TNF-beta	Tumor necrosis factor beta	Human	Growth factor	ORF Genetics
VEGF	Vascular endothelial growth factor	Human	Growth factor	ORF Genetics
VEGF	Vascular endothelial growth factor	Mouse	Growth factor	ORF Genetics
FaeG F4	Fimbrial adhesion	*E. coli*	Vaccine	Joensuu et al. (2006)
	Anti glycophorin scFv-HIV epitope fusion	Synthetic	Diagnostic	Schünmann et al. (2002)
Antithrombin III	Antithrombin III	Human	Proteinase inhibitor	Stahl et al. (2002)
α1-Antitrypsin	α1-Antitrypsin	Human	Proteinase inhibitor	Stahl et al. (2002)
HAS	Serum albumin	Human	Carrier protein	Stahl et al. (2002)
Lactoferrin	Lactoferrin	Human	Carrier protein	Stahl et al. (2002), Kamenarova et al. (2007)
Lysozyme	Lysozyme	Human	Enzyme	Stahl et al. (2002), Huang et al. (2006)
Glucanase	(1,3-1,4)-β-glucanase	Bacillus species	Glucan modifying enzyme	Horvath et al. (2000)
Hb	Haemoglobin	Vitreoscilla	Carrier protein	Wilhelmson et al. (2007)

(continued)

Table 13.1 (continued)

Recombinant protein	Full name of protein	a.a. sequence	Protein type	Source
Thaumatin	Thaumatin	*Thaumatococcus daniellii*	Sweetener	Stahl et al. (2009)
rCla1	Collagen 1a	Human	Structural protein	Ritala et al. (2008), Eskelin et al. (2009)

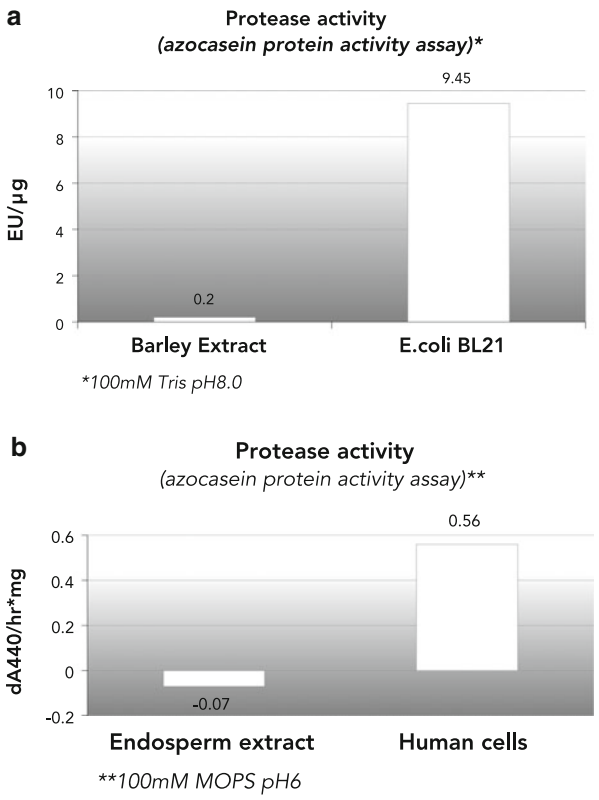

Fig. 13.3 Proteolytic activity of barley, bacteria (**a**) and human cells (**b**). Azocasein-containing solutions were incubated with clarified extracts of milled grain and lysates of *E. coli* (BL21) and human neutrophil cells. After precipitation, the absorbance of the released soluble azopeptides in the supernatant indicates the extent of proteolytic activity in the samples

used for the expression of recombinant proteins, and lysed human mononuclear cells using the azocasein proteolytic assay (Millet 1977) (Fig. 13.3). The results confirm very low proteolytic activity in the barley grain extract which is minimal compared to metabolically active bacterial and human cells. This suggests that a recombinant protein accumulated in barley grains is less likely to be exposed to proteolytic activity than in metabolically active bacterial or mammalian cells. This would contribute to the stability of recombinant proteins stored within the grains.

Recombinant growth factors purified from barley grains have been observed in cell proliferation bioassays to fully maintain their activity in a 2-year-old grain

material (ORF Genetics, unpublished results), indicating the stability of recombinant proteins stored in grain tissue.

It is, therefore, not unreasonable to expect that recombinant proteins accumulated amongst grain storage proteins can remain stable for decades if intact grains are stored under optimal conditions.

13.5 Safety and Quality of Products

Endotoxins are bacteria-derived molecules, e.g. lipopolysaccharides and lipooligosaccharides, that induce signalling cascades for pro-inflammatory cytokines in mammalian cells causing damage and stress to cell cultures. Depending on the cell line, this may jeopardise the cell culture and affect research results and cell-based production of biologicals (Epstein et al. 1990; Lieder et al. 2013). Endotoxins are, thus, a major concern for contamination of bacterially produced recombinant proteins used in cell culture. For pharmaceutical production, it is essential to verify that the recombinant product is not contaminated with bacterial endotoxins that may otherwise cause inflammation and disease, or even endotoxic shock in patients. Plants do not produce endotoxins, and recombinant proteins produced within grains should therefore be void of endotoxins. To verify the expected low endotoxin content of purified, freeze-dried plant-derived recombinant human growth factors, VEGF and IFN-gamma, they were subjected to endotoxin measurements by a validated commercial laboratory (Associates of Cape Cod, Inc.) (Fig. 13.4).

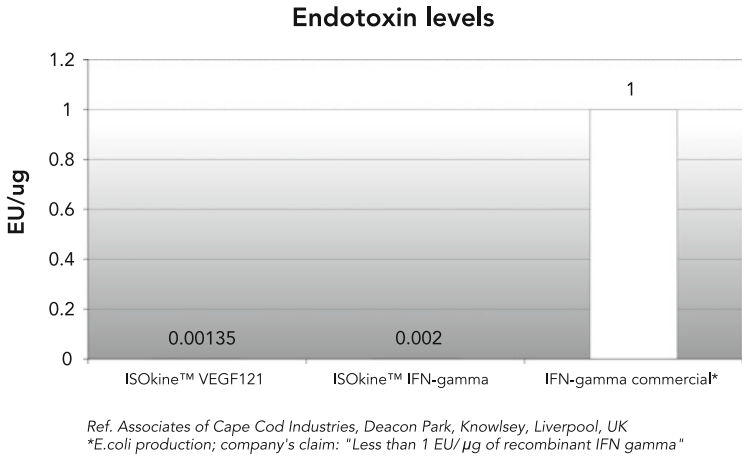

Fig. 13.4 Endotoxin measurements. *Limulus* amebocyte lysate (LAL) tests detect and quantify bacterial endotoxins extracted from the outer membrane of gram-negative bacteria. The samples were subjected to turbidimetric LAL testing (Associates of Cape Cod, Intl, Inc., Deacon Park, Moorgate Road, United Kingdom). In short, the results showed that endotoxin content in barley-derived products was hardly detectable with the most sensitive endotoxin assays

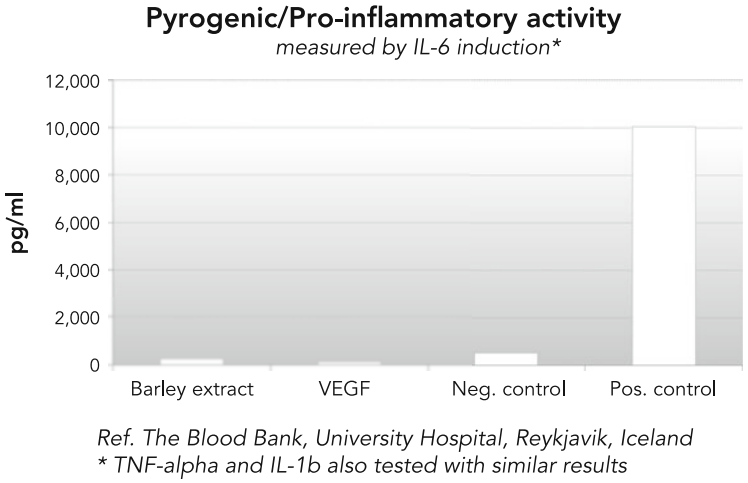

Fig. 13.5 Monocyte activation test (MAT assay) was performed with crude barley extract and a mock sample of non-transgenic barley extract that underwent the full purification protocol for a growth factor (VEGF). Human mononuclear cell cultures of density 1×10^6 were supplemented with various concentrations of the barley samples, negative control (cells without supplement) and positive control cell culture supplemented with 50 ng lipopolysaccharide (LPS). The release of the pyrogenic marker interleukin-6 (IL-6), shown above, along with eight other cytokines (data not shown) was assayed using the Procarta Cytokine Assay Kit, Human 10-plex (Affymetrix, 3420 Central Expressway Santa Clara, CA 95051, USA) monitored with a Luminex 100 instrument (the Blood Bank, Snorrabraut 60,105 Reykjavík, Iceland)

The possibility of pyrogenic, pro-inflammatory compounds being present in barley grain extracts was examined. The Monocyte Activation Test (MAT assay) measures the induction of pyrogenic marker cytokines in human monocytes. To further validate barley as a suitable host for cytokine production, the barley background was studied by the MAT assay (Fig. 13.5).

Neither barley extract nor purified barley grain-derived VEGF mock fraction induced any pyrogenic response indicating strongly that barley grain as a host tissue for expression of recombinant proteins does not carry inherent pyrogenic compounds detrimental to cell culture. This is yet another indication that barley grain as a source for recombinant proteins can be considered and generally recognised as safe.

To determine the biological activity of recombinant protein purified from barley grain, the proteins are subjected to cell-based bioassays.

The Flt3 ligand produced in barley endosperm and purified from transgenic barley grains was previously shown to exhibit yields comparable to bacterial expression systems and to be biologically functional in cell-based assays (Erlendsson et al. 2010). Fibroblast growth factor (FGF basic) was produced in a barley endosperm and purified from transgenic barley grains, and the activity was assessed with a cellular proliferation assay on FGF basic responsive, mouse 3T3 cells. Serial dilutions of the recombinant human FGF basic proteins in assay media

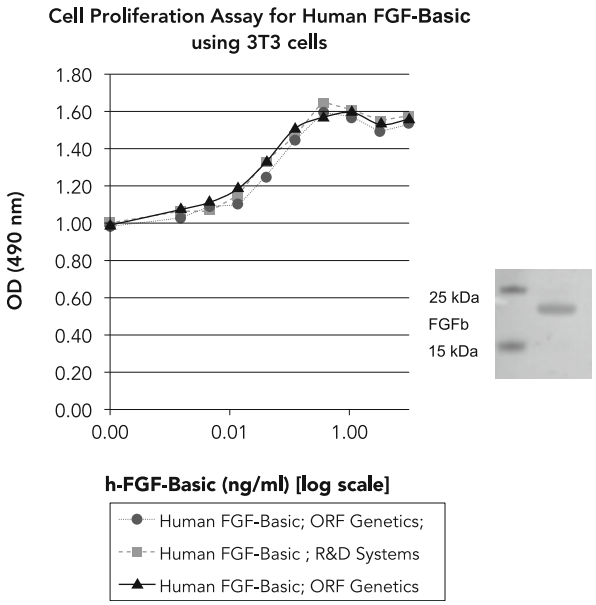

Fig. 13.6 Results of bioactivity assay of plant-derived recombinant human FGF basic growth factor purified from the grains of transgenic barley. The graph shows a cellular proliferation bioassay exposing FGF basic responsive mouse 3T3 cells to dilution series of the FGF basic protein (SBH Sciences, Natick, MA, USA). Two samples of plant-derived FGF basic (ORF Genetics, Víkurhvarf 3, 203 Kópavogur, Iceland) are compared to *E. coli*-derived recombinant human FGF basic (R&D systems; Cat# 233-FB/CF). Next to the graph is a Coomassie-stained gel showing purified plant-derived FGF basic next to molecular size markers 15 kDa and 25 kDa to the *left*

were added to cell culture and incubated for 42 h at 37 °C before staining and measuring the optical density at 490 nm (SBH Sciences, Natick, MA, USA) (see Fig. 13.6).

The results show that the recombinant human FGF basic protein produced in barley is as active as the leading *E. coli*-derived recombinant human FGF basic available on the market. A benefit of the eucaryotic plant expression systems is that human proteins are expressed, folded and processed in the same way in plants and humans, maintaining structure and function with only minor differences in post-translational modifications of proteins. In comparison, bacterial expression systems are unable to do most post-translational modifications and frequently discard recombinant human proteins into inclusion bodies requiring costly and cumbersome refolding of the denatured proteins to regain activity. The lack of post-translational modifications such as glycosylation can affect the assembly, stability, half-life and activity of recombinant proteins, as shown by the analysis of Runkel et al. (1998) on the activity and stability of glycosylated and deglycosylated forms of the growth factor IFN-beta. The deglycosylated form showed less activity and was more prone to thermal denaturation. Post-translational modifications can thus

be important for the proper function of biologically active proteins. Although plants are able to glycosylate proteins in a similar manner as mammalians, subtle differences exist. Plants lack sialic acid from their glycan structures, while the plant-specific glycans β1,2-xylose and core α1,3-fucose residues are absent in mammalian glycoproteins. Both xylose and α1,3-fucose have been suggested to have allergenic properties and might therefore be expected to be problematic to plant-based pharmaceuticals (Bardor et al. 2003). Advancements in glycoengineering may bring solutions to such problems (Gomord et al. 2010).

Such concerns are specifically addressed in safety studies during clinical trials. In fact, the first plant-made pharmaceutical glucocerebrosidase is glycosylated and carries both xylose and fucose and has not been observed to cause adverse effects beyond what is to be expected in enzyme replacement therapy (Zimran et al. 2011). The fact that it passed the clinical trials and received market authorisation as an injectable drug indicates that suggested allergenicity of plant glycans is at least not a general phenomenon, but is to be studied on a case-by-case basis, as is the case with any new pharmaceuticals in development.

Molecular farming with barley as host system has become quite extensive in terms of recombinant products and is the result of intensive research and development within academia research institutes and companies during the last two decades. Barley today is probably the molecular farming platform that has delivered the highest number of plant-made recombinant proteins to the market. Table 13.1 gives an overview of recombinant proteins that have been produced in barley for molecular farming purposes. Growth factors are prominent on the list as a result of the focus of one company on the family of growth factors and cytokines as mentioned. They are used in just about every aspect of cell biology, immunology, stem cell research and medical research that involves cell culture, such as regenerative medicine as well as biopharmaceutical development. Recently published results by Ritala et al. (2014) describe the expression and accumulation of an antibody (IgE) in grains. A recent review of the use of barley for production of recombinant proteins can be found in Magnusdottir et al. (2013).

Conclusion

Barley is, in many respect, extraordinary well suited for contained molecular farming operations; the agricultural features and human dependency with the limited fitness, containment through self-pollination and heavy grains provide for management of cultivation. Although time-consuming, once stable transformant elite lines have been established, the grain-specific expression together with the protein storage properties of grains offers flexibility in production operations, such as stockpiling of harvest and just-in-time processing catering to the demand. Barley grain-based products are inherently of higher quality than products obtained with traditional expression hosts, i.e. bacteria and mammalian cells, being animal-free, serum-free and

(continued)

endotoxin-free. They are void of human or animal infectious agents and low in pyrogenic and pro-inflammatory activity, the proteins are naturally folded by the plants eucaryotic protein-folding mechanism and there is no risk of contamination by other endogenous mammalian proteins.

Molecular farming is, in many aspects, already competitive with more traditional expression systems that have been pressed to their limits in efficiency as a result of decades of intensive optimisation. The prospect of all the unlocked potential and optimisation that this novel green manufacturing technology has in reserve for the future to further improve expression levels, purification yields, and process optimisation is truly encouraging. Ongoing research efforts in barley genomics, proteomics and glycomics will pave the way for further improvements of molecular farming with barley. Some of the unharnessed potential undoubtedly includes improving further the expression levels with identification of even more efficient tissue-specific promoters, postharvest inducible promoters, regulatory sequences, responsive elements and stabilising elements. Tailoring of post-translational modification and intracellular protein targeting are likely to add to the versatility of the barley system. Advances in genetic transformation of barley varieties and cultivation of elite barley lines of selected barley varieties under optimised conditions for protein accumulation will help harness the potential of upstream processes, while advances in downstream processes and protein purification will continue to improve the yields and efficiency of molecular farming in barley.

The time is ripe to weld the efforts of basic and applied science to strengthen the foundation of a green, sustainable manufacturing technology and secure the harvest of some of nature's most complex and valuable compounds—proteins.

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References

Affymetrix. http://www.medprobe.com/files//2009%20spring%20Procarta.pdf. Cited May 2013

Associates of Cape Cod Inc. http://www.acciusa.com/cts/test/index.html. Cited 5 June 2013

Bardor M, Faveeuw C, Fitchette A-C, Gilbert D, Galas L, Trottein F, Faye L, Lerouge P (2003) Immunoreactivity in mammals of two typical plant glyco-epitopes, core a(1,3)-fucose and core xylose. Glycobiology 13(6):427–434. doi:10.1093/glycob/cwg024

Basaran P, Rodríguez-Cerezo E (2008) Plant molecular farming: opportunities and challenges. Crit Rev Biotechnol 28:153–172

Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, Murray E, Morck D, Moloney MM (2010) Seed-based expression systems for plant molecular farming. Plant Biotechnol J 8(5):588–606

Canadian Seed Growers' Association (2013) Circular 6, Table 2.4.2, Revision 1.8.2013, pp 2–6
Caspers MPM, Lok F, Sinjorgo KMC, van Zeijl MJ, Nielsen KA, Cameron-Mills V (2001) Synthesis, processing and export of cytoplasmic endo-1,4-xylanase from barley aleurone during germination. Plant J 26:191–204
Cho MJ, Choi HW, Jiang W, Ha CD, Lemaux PG (2002) Endosperm-specific expression of green fluorescent protein driven by the hordein promoter is stably inherited in transgenic barley (Hordeum vulgare) plants. Physiol Plant 115:144–151
Christou P, Stoger E, Twyman RM (2008) Monocot expression systems for molecular farming. In: Fersht A (ed) Protein science encyclopedia. Wiley, New York. doi:10.1002/9783527610754.tr02
Drake PM, Chargelegue DM, Vine ND, van Dolleweerd CJ, Obregon P, Ma JK (2003) Rhizosecretion of a monoclonal antibody protein complex from transgenic tobacco roots. Plant Mol Biol 52(1):233–241
Epstein J, Kelly CE, Lee MM, Donahue PK (1990) Effect of *E.coli* endotoxin on mammalian cell growth and recombinant protein production. In Vitro Cell Dev Biol 26:1121–1122
Erlendsson LS, Muench MO, Hellman U, Hrafnkelsdóttir SM, Jonsson A, Balmer Y, Mäntylä E, Orvar BL (2010) Barley as a green factory for the production of functional Flt3 ligand. Biotechnol J 5(2):163–171
Eskelin K, Ritala A, Suntio T, Blumer S, Holkeri H, Wahlström EH, Baez J, Mäkinen K, Maria NA (2009) Production of a recombinant full-length collagen type I alpha-1 and of a 45-kDa collagen type I alpha-1 fragment in barley seeds. Plant Biotechnol J 7:657–672
Fujiwara Y, Aiki Y, Yang L, Takaiwa F, Kosaka NM (2010) Extraction and purification of human interleukin-10 from transgenic rice seeds. Protein Expr Purif 72:125–130
Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 8(5):564–587. doi:10.1111/j.1467-7652.2009.00497.x
Han J, Lakshman DK, Galvez LC, Mitra S, Baenziger PS, Mitra A (2012) Transgenic expression of lactoferrin imparts enhanced resistance to head blight of wheat caused by Fusarium graminearum. BMC Plant Biol 12:33. doi:10.1186/1471-2229-12-33
Hensel G (2011) Genetic transformation of Triticeae cereals for molecular farming. In: Alvarez M (ed) Genetic transformation. InTech, Rijeka, pp 171–192. doi:10.5772/868, http://www.intechopen.com/books/genetic-transformation
Hensel G, Valkov V, Middlefell-Williams J, Kumlehn J (2008) Efficient generation of transgenic barley: the way forward to modulate plant-microbe interactions. J Plant Physiol 165:71–82
Hensel G, Himmelbach A, Chen W, Douchkov DK, Kumlehn J (2011) Transgene expression systems in the Triticeae cereals. J Plant Physiol 168:30–44
Hermannsson J, Kristjansdottir TA, Stefansson TS, Hallsson JH (2010) Measuring gene flow in barley fields under Icelandic sub-arctic conditions using closed-flowering varieties. Icel Agric Sci 23:51–59
Horvath H, Huang J, Wong O, Kohl E, Okita T, Kannangara CG, von Wettstein D (2000) The production of recombinant proteins in transgenic barley grains. Proc Natl Acad Sci U S A 97(4):1914–1919
Huang N, Rodriguez RL, Hagie FE (2006) Expression of human milk proteins in transgenic plants. US Patent 7,718,851
Joensuu JJ, Kotiaho M, Teeri TH, Valmu L, Nuutila AM, Oksman-Caldentey KM, Niklander-Teeri V (2006) Glycosylated F4 (K88) fimbrial adhesin FaeG expressed in barley endosperm induces ETEC-neutralizing antibodies in mice. Transgenic Res 15(3):359–373
Kamenarova K, Abumhadi N, Gecheff K, Atanassov A (2005) Molecular farming in plants: an approach of agricultural biotechnology. J Cell Mol Biol 4:77–86
Kamenarova K, Gecheff K, Stoyanova M, Muhovski Y, Anzai H, Atanassov A (2007) Production of recombinant human lactoferrin in transgenic barley. Biotechnol Biotech Equip 21(1):18–27
Lieder R, Gaware VS, Thormodsson F, Einarsson JM, Ng CH, Gislason J, Masson M, Petersen PH, Sigurjonsson OE (2013) Endotoxins affect bioactivity of chitosan derivatives in cultures of

bone marrow-derived human mesenchymal stem cells. Acta Biomater 9(1):4771–4778. doi:10.1016/j.actbio.2012.08.043

Ma JK, Barros E, Bock R, Christou P, Dale PJ, Dix PJ, Fischer R, Irwin J, Mahoney R, Pezzotti M, Schillberg S, Sparrow P, Stoger E, Twyman RM (2005) Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants. EMBO Rep 6(7):593–599

Magnusdottir A, Vidarsson H, Björnsson JM, Örvar BL (2013) Barley grains for the production of endotoxin-free growth factors. Trends Biotechnol 31(10):572–580

Millet J (1977) Characterization of a protein inhibitor of intracellular protease from Bacillus subtilis. FEBS Lett 74:59–61

Nair SK, Wang N, Turuspekov Y, Pourkheirandish M, Sinsuwongwat S, Chen G, Sameri M, Tagiri A, Honda I, Watanabe Y, Kanamori H, Wicker T, Stein N, Nagamura Y, Matsumoto T, Komatsuda T (2010) Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. Proc Natl Acad Sci U S A 107(1):490–495

Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, Mejima M, Nakanishi U, Matsumura A, Uozumi A, Hiroi T, Morita S, Tanaka K, Takaiwa F, Kiyono H (2007) Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proc Natl Acad Sci U S A 104(26):10986–10991

ORF Genetics. http://orfgenetics.com/. Cited 9 July 2013

Patel M, Johnson JS, Brettell RIS, Jacobsen J, Xue GP (2000) Transgenic barley expressing a fungal xylanase gene in the endosperm of the developing grains. Mol Breed 6:113–123

Penney CA, Thomas DR, Deen SS, Walmsley AM (2011) Plant-made vaccines in support of the millennium development goals. Plant Cell Rep 30(5):789–798

Protalix Inc. http://www.protalix.com/product-development/elelyso.asp. Cited 9 July 2013

Ramessar K, Capell T, Christou P (2008a) Molecular pharming in cereal crops. Phytochem Rev 7:579–592. doi:10.1007/s11101-008-9087-3 (Springer, Berlin)

Ramessar K, Sabalza M, Capell T, Christou P (2008b) Maize plants: an ideal production platform for effective and safe molecular pharming. Plant Sci 174:409–419

Ritala A, Nuutila AM, Aikasalo R, Kauppinen V, Tammisola J (2002) Measuring gene flow in the cultivation of transgenic barley. Crop Sci 42(1):278–285

Ritala A, Wahlström EH, Holkeri H, Hafren A, Mäkeläinen K, Baez J, Mäkinen K, Nuutila AM (2008) Production of a recombinant industrial protein using barley cell cultures. Protein Expr Purif 59(2):274–281

Ritala A, Leelavathi S, Oksman-Caldentey VS, Reddy K-M, Laukkanen M-L (2014) Recombinant barley-produced antibody for detection and immunoprecipitation of the major bovine milk allergen, b-lactoglobulin. Transgenic Res 23(3):477–87. doi:10.1007/s11248-014-9783-2

Runkel L, Meier W, Pepinsky RB, Karpusas M, Whitty A, Kimball K, Brickelmaier M, Muldowney C, Jones W, Goelz SE (1998) Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (IFN-beta). Pharm Res 15 (4):641–649

SBH Sciences, Natick, MA, USA. http://www.sbhsciences.com/

Schünmann PHD, Coia G, Waterhouse PM (2002) Biopharming the SimpliRED™ HIV diagnostic reagent in barley, potato and tobacco. Mol Breed 9(2):113–121

Sharma AK, Sharma MK (2009) Plants as bioreactors: recent developments and emerging opportunities. Biotechnol Adv 27:811–832

Stahl R, Horvath H, Van Fleet J, Voetz M, von Wettstein D, Wolf N (2002) T-DNA integration into the barley genome from single and double cassette vectors. Proc Natl Acad Sci U S A 99 (4):2146–2151

Stahl R, Luhrs R, Dargatz H (2009) Thaumatin from transgenic barley. US Patent Application, US 2009/0031458

Steiner AM, Ruckenbauer P (1995) Germination of 110-year-old cereal and weed seeds, the Vienna Sample of 1877. Verification of effective ultra-dry storage at ambient temperature. Seed Sci Res 5:195–199

Stoger E, Ma JKC, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16:167–173

Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2–15

The Blood Bank, Landspitali University Hospital, Snorrabraut 60, Reykjavik 105, Iceland

USDA (2006) NEPA 05-340-01r. http://www.aphis.usda.gov/brs/aphisdocs/05_34001r_ndd.pdf

Vickers CE, Xue G, Gresshoff PM (2006) A novel cis-acting element, ESP, contributes to high level endosperm-specific expression in an oat globulin promoter. Plant Mol Biol 62:195–214

Wilhelmson A, Kallio PT, Oksman-Caldentey KM, Nuutila AM (2007) Heterologous expression of Vitreoscilla haemoglobin in barley (Hordeum vulgare). Plant Cell Rep 26(10):1773–1783

Yano M, Hirai T, Kato K, Hiwasa-Tanase K, Fukuda N, Ezura H (2010) Tomato is a suitable material for producing recombinant miraculin protein in genetically stable manner. Plant Sci 178:469–473

Zeder MA, Emshwiller E, Bradley DG, Smith BD (2006) Documenting domestication: the intersection of genetics and archaeology. Trends Genet 22(3):139–155

Zimran A, Brill-Almon E, Chertkoff R, Petakov M, Blanco-Favela F, Muñoz ET, Solorio-Meza SE, Amato D, Duran G, Giona F, Heitner R, Rosenbaum H, Giraldo P, Mehta A, Park G, Phillips M, Elstein D, Altarescu G, Szleifer M, Hashmueli S, Aviezer D (2011) Pivotal trial with plant cell-expressed recombinant glucocerebrosidase, taliglucerase alfa, a novel enzyme replacement therapy for Gaucher disease. Blood 118(22):5767–5773. doi:10.1182/blood-2011-07-366955

Part II
Recent Progress in Methodology

Chapter 14
Development of Sequence Resources

Nils Stein

14.1 Introduction

Access to a genome sequence is now considered a prerequisite for efficient studies on the biology of an organism. A genetically and physically anchored genome sequence provides direct access to all genes and corresponding regulatory sequences that modulate gene expression and leads to a better understanding of species-specific characteristics and traits. A gap-free reference sequence—the principle objective of most biological communities—reveals the coding and regulatory nucleotide sequence in the genomic environment, the grey matter in which the functional genes and their control elements are embedded and which, in the case of large and complex genomes, contributes the major part of the genome sequence. Understanding the contribution and impact that genomic context makes to the dynamics of a genome and to the expression of traits is in its infancy. Nevertheless, even access to partial genome sequence information is highly enabling for the development of new tools in applied crop research and crop improvement.

The genome of barley is estimated to contain 5.1 billion base pairs (Doležel et al. 1998) and contains over 80 % of repetitive DNA (IBSC 2012; Wicker et al. 2009). As a direct consequence, progress in the whole-genome sequencing in barley has lagged behind that achieved for small genome plant species as well as in economically more important crop species such as maize that has smaller but comparably complex genomes (Feuillet et al. 2011). Put simply, whole-genome sequencing of the barley genome was considered just too expensive if it was to rely on traditional Sanger sequencing technology, especially given the commercial importance of the crop and size of its research community. However, when next-generation sequencing (NGS) technologies appeared with their promise of a

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Table 14.1 Access to important sequence resources of barley

Type of sequence data	URL
Fl-cDNA	http://barleyflc.dna.affrc.go.jp/hvdb/
EST assemblies	http://www.harvest-web.org/ http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=barley
WGS assembly and physical map	http://webblast.ipk-gatersleben.de/barley/ http://mips.helmholtz-muenchen.de/plant/barley/index.jsp
BAC end sequences, BACs, sorted chromosomes, RNAseq	For accession numbers, please refer to the supplemental information of IBSC (2012)

dramatic decrease in sequencing costs (Mardis 2008; Service 2006), the International Barley Genome Sequencing Consortium (IBSC) was formed to establish a practical working agenda towards physical mapping and sequencing of the barley genome (http://barleygenome.org, Schulte et al. 2009).

This chapter provides both personal and historical overview of the development of the diverse sequence resources of barley (Table 14.1) that accumulated both before and since the IBSC was initiated. The resources themselves allow key insights into the barley genome but more importantly provide new opportunities and perspectives for application in the context of barley crop improvement.

14.2 Sanger Sequencing-Based Resources

14.2.1 Genetic Marker Sequences

Early DNA marker maps in barley were developed by the use of Southern hybridisation-based restriction fragment length polymorphism (RFLP) markers. These markers proved very useful for genetic mapping and were employed to develop the first comprehensive molecular marker maps in barley (Graner et al. 1991; Kleinhofs et al. 1993). The use of hybridisation-based markers stimulated comparative mapping in related grass genomes and helped to establish the early maps of grass genome collinearity (Moore et al. 1995). They also established the value of the relatively small (340 Mbp) rice genome as a reference for the bigger grass genomes (Bennetzen and Freeling 1993, 1997) and initiated the concept of 'conserved synteny' amongst related grass genomes. However, from an applied point of view, hybridisation-based markers were never exemplary tools for marker-assisted selection due to the laborious procedures and the need to label probes with radioisotopes. With the establishment of polymerase chain reaction (PCR) as a standard technology for diagnostic applications, there was the aim of converting laborious RFLP markers into convenient PCR assays. RFLP markers, which represented genomic DNA fragments cloned into plasmid vectors, were subjected to systematic sequencing. As an example for the 'MWG' RFLP marker collection,

almost 500 sequence tags have been submitted to NCBI gene bank (Michalek et al. 1999) which allows to use them as sequence-tagged site (STS) markers. Despite this effort, the combination of low levels of observed polymorphism and the emergence of powerful alternative PCR-based technologies such as AFLPs (Becker et al. 1995; Qi et al. 1998) and SSRs (Ramsay et al. 1999) effectively sidelined the use of STS derived from RFLPs for use in genetic studies.

14.2.2 Sequencing Expressed Genes

Already at the time of RFLP mapping, molecular markers were developed preferentially from expressed genes, i.e. cDNA (complementary DNA obtained by reverse transcription of mRNA). This was generally because cDNA probes usually revealed low- or single-copy markers and could be transferred between grass species with reasonably high efficiency. Indeed, the International Triticeae Mapping Initiative (ITMI, http://wheat.pw.usda.gov/ITMI/), established in 1989, identified the importance of cDNA sequences as a source of efficient and potentially higher throughput molecular markers in Triticeae species. Members of ITMI initiated the International Triticeae EST Consortium in 1999 that provided a concerted effort focused on sequencing cDNA clones of Triticeae species, mainly barley and wheat. Within a few years, over 500,000 barley ESTs were submitted to public databases (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). These EST sequences were then assembled to derive consensus sequences that represented the 'unigene' set from the respective species (e.g. Zhang et al. 2004). The Gene Index Project provides access to a barley EST assembly (DFCI barley gene index, http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=barley) combining 502,606 ESTs into 43,310 tentative consensus (TC) sequences and 39,494 singleton ESTs. An alternative set of TC sequences of barley is provided via the HarvEST project (http://www.harvest-web.org/) at UC, Riverside, USA. Six assemblies with incrementally increasing numbers of input EST sequences (starting from 349,700 ESTs in assembly 21 up to 519,726 ESTs in assembly 36) were generated at either 'stringent' or 'relaxed' assembly criteria considering also sequence quality information. The various HarvEST assemblies were identified between 23,000 and 32,000 TC sequences.

The EST assembly 21 of the HarvEST project (HarvEST 21) was used as a template for the design and development of an Affymetrix gene expression microarray of barley (22K Barley 1 GeneChip, Close et al. 2004) which has been used widely by the barley community for gene expression analyses of specific tissues (Sreenivasulu et al. 2008) and developmental stages (Druka et al. 2006) or as a tool for monitoring differential gene expression in response to biotic or abiotic stress (Svensson et al. 2006; Wise et al. 2007b). A substantial amount of the data generated in such studies has been collected in specialised databases like PLEXdb (http://www.plexdb.org/, Wise et al. 2007a) and GENEVESTIGATOR (https://www.genevestigator.com/gv/doc/plant/content.jsp). These now provide a point of

entry into data sets that summarise the patterns of expression of any researcher's favourite candidate gene with the proviso that is represented on the Affymetrix microarray platform.

Recently, the large public EST resource has been complemented by a comprehensive set of almost 25,000 full-length cDNA (fl-cDNA) sequences (Matsumoto et al. 2011). Even though a certain fraction of the represented transcripts still may lack the 5′-untranslated region (UTR), the majority of the sequences provide direct access to all exons of the corresponding genes. Consequently, these sequences are an exceptionally valuable tool for annotating genes in the context of sequencing the whole barley genome (see below).

In line with the original goal of ITEC, the available EST resources have been extensively exploited for the development of dense gene-based marker (transcript) maps. EST sequences can be easily screened for the presence of simple sequence repeat (SSR) sequences (Thiel et al. 2003) which can be converted into PCR-based markers (Varshney et al. 2007). Based on RFLP, single nucleotide polymorphism (SNP) and SSR markers, a first 1,000-loci transcript map provided refined insights into conserved synteny amongst the grasses and allowed traces of ancient genome duplication in barley to be revealed (Stein et al. 2007; Thiel et al. 2009). As described, ESTs were also the template for the development of the Affymetrix Barley 1 microarray platform. When this was used to assay RNA isolated from comparable tissues from all individuals in a biparental segregating population, significant differences in levels of gene expression amongst individuals were used by Potokina et al. (2008) to construct a gene-based genetic linkage map. By surveying the expression of some 16,000 transcripts, they were able to robustly locate almost 6,000 genes as transcript-derived markers, or TDMs, on the barley genetic map. However, TDMs themselves are not generally considered suited to routine genetic analysis. A high-throughput SNP platform for barley genotyping was developed on the basis of the HarvEST32 assembly which allowed almost 3,000 loci to be positioned on a consensus map derived from four populations (Close et al. 2009). This map was recently revised by integrating further mapping data from an additional six populations (Muñoz-Amatriaín et al. 2011) and provided for some years a reference set of markers for germplasm characterisation and mapping in barley. A comprehensive transcript map, comprising almost 3,000 loci and derived from mapping in a single population, was developed on the basis of the above-mentioned fl-cDNA data set (Sato et al. 2009). Finally and more recently, a SNP platform comprised of approximately 8,000 SNPs has been developed and extensively utilised by the barley genetics community (Comadran et al. 2012).

14.2.3 BAC End Sequences

Sequencing the paired ends of large genomic DNA inserts of cosmid/fosmid or bacterial artificial chromosome (BAC) clones is a very important task during genome sequencing projects. Such paired sequence tags combined with knowledge

of the physical distance separating them in the genome of interest help 'scaffold' independent sequence contigs across stretches of repetitive DNA that would otherwise be difficult to bridge by de novo shotgun sequencing and assembly. BAC end sequences (BESs) also generate a relatively unbiased overview of genome composition of a species and can be exploited for the development of molecular markers (Paux et al. 2006). In barley a large effort has been made to sequence the end sequences of over 360,000 BAC inserts (571,814 BES, cumulative 373.5 Mbp) to provide sequence tags for anchoring the barley physical map to the genetic map. As may have been expected from the complexity of the barley genome, only 29 % of the sequences resided in non-repetitive DNA and thus provided sequence tags that proved useful for anchoring other sequence resources or genetic markers (The International Barley Genome Sequencing Consortium, IBSC 2012). However, BES still provided the most important resource for integrating other sequences—like >300 Mbp of whole-genome shotgun sequence contigs—into an anchored physical/genetic map of the barley genome (IBSC 2012). Barley BES data can be accessed for sequence comparisons at http://webblast.ipk-gatersleben.de/barley/.

14.3 Next-Generation Sequencing-Based Resources

14.3.1 Genomic Survey Sequencing

The first NGS platforms available on the market provided high throughput but only short-sequence read output (100 nucleotides (nt) for Roche/454 GS20, 35 nt for Illumina GA) if compared to traditional Sanger sequencing. Such technology would not, at that stage of development, allow whole-genome shotgun sequencing and de novo assembly of any plant genome; however, the technology was initially designed for other purposes. The main application at the time was to allow re-sequencing of a larger number of samples by aligning (map) the short reads to a reference sequence (i.e. the human genome). However, the new opportunities potentially offered by NGS methodologies were not lost on the international barley genomics community, invoking a number of pilot studies. This was because—even at shallow sequence coverage of about 1 % of a haploid barley genome equivalent generated using Roche/454 GS20 technology—many general characteristics of the genome could be captured. Thus, approximately 60 % of the sequences could be assigned to known repetitive DNA elements, while 10 % of the data could eventually be assigned to previously uncharacterised elements of the barley genome (Wicker et al. 2009). Furthermore, by comparing the composition of the repetitive DNA sequences to individually sequenced BAC clones, predictions could be made for the distribution of retroelements and DNA transposons in the barley genome. In addition by expanding this comparison to sequenced BAC clones from the closely related diploid wheat *Triticum monococcum*, it could be shown that some families

of repetitive DNA elements populating the barley genome in large numbers are absent in *T. monococcum* and vice versa (Wicker et al. 2009).

The second publicly available NGS platform, Illumina Genome Analyzer (GA), provided in its early version shorter reads (35 nt) at even higher sequence output. While this wasn't suitable for genome sequence assembly, the data—similar to the above-mentioned GS20 data set—could be used effectively to assemble statistical characteristics of barley genome composition. Almost 600 Mbp of raw sequence data were generated from genomic DNA (equivalent to about 10 % of a haploid barley genome coverage) (Wicker et al. 2008). From the sequence reads, all possible 20-mers were derived from a sliding window analysis using 1-nt steps. All of the resulting 20-mers were then counted, and an index of mathematically defined repeats (MDR index, Kurtz et al. 2008) of each 20-mer was built to provide an accurate estimation of copy-number statistics (Wicker et al. 2008). This MDR index could then be used to annotate any barley genomic sequence into unique and repetitive regions.

14.3.2 BAC Clone Sequencing

Before the availability of NGS technology, large contiguous genomic sequences of barley were rare and were generated mainly in the course of map-based cloning of specific target genes [previously reviewed in Stein (2007); Eversole et al. (2009)]. It was therefore important to test the applicability of short-read NGS technology for sequencing and assembling large insert BAC genomic clones, particularly since the vision for sequencing of the 5 Gbp barley genome relied to large extent on hierarchical sequencing of the minimum tiling path of overlapping BACs selected from a physical map (Eversole et al. 2009; Schulte et al. 2009). In an early study, four BAC clones with reference Sanger sequence available were selected for re-sequencing on the Roche/454 GS20 platform (Wicker et al. 2006). This illustrated the potential of using NGS to sequence a large and complex genome carrying 80 % or more of repetitive DNA. Due to the short-read length, de novo assembly of the sequence data produced high numbers of relatively small contigs; however, low-copy sequences like genes assembled easily (Wicker et al. 2006). This approach was further elaborated by taking account of the high overall sequencing capacity of individual NGS sequencing runs. Multiple BAC clones have now been individually labelled after mechanical shearing with unique sequence tags ['barcodes' or multiplex identifiers (MID)] and pooled in batches of 48 clones per partition on a picotiter plate of the Roche/454 GS FLX system (Steuernagel et al. 2009). The longer reads of the FLX platform in combination with the use of different assembly tools revealed that sequence data obtained by this strategy was almost comparable to Sanger-based shotgun sequencing in terms of the assembly quality obtained. Adding more mate-pair sequence data helped to scaffold the obtained contig sequences in their physical order and thus led to further

improvements of the assembly (Taudien et al. 2011). Since these pilot studies, sequencing throughput and associated costs have now decreased by over a factor of 10.

The need to use barcode sequence tags to allow sorting of sequence data according to the individual input DNAs introduces a substantial amount of labour to the sequencing procedure. To avoid this step, it could be shown that pooling of BAC clones in a combinatorial design and sequencing to very high coverage on Illumina GAIIx or HiSeq allow subsequent deconvolution of sequence reads and assembly according to their origin. Up to 70 % of the original BAC inserts can be reliably assembled into large contigs in a clone-specific manner from such non-barcoded pools (Lonardi et al. 2012). To date more than 6,000 barley BAC clones from the physical map have been sequenced and assembled (IBSC 2012; Lonardi et al. 2012). BAC sequences obtained by 454 sequencing of barcoded pools can be accessed for sequence search at http://webblast.ipk-gatersleben.de/barley/viroblast.php. Sequences assembled from combinatorial pool sequencing are accessible via http://www.harvest-web.org/utilmenu.wc?job=RTRVFORM&db=MOREX_HV3_9. An additional set of 400 BAC clones from a Japanese cultivar 'Haruna Nijo' genetically assigned to chromosome 3H were sequenced as untagged pools on the Roche/454 GS20 and FLX systems (Sato et al. 2011). Current efforts are focused on sequencing complete individual barley chromosomes to reference standard (N. Stein and R. Waugh, pers. comm.)

14.3.3 Survey Sequencing of Flow-Sorted Chromosomes

The genome size of barley and other Triticeae species is a disadvantage for whole-genome sequencing. Due to a low basic chromosome number ($N = 7$), the large genome size, however, translates into large chromosome size (average size = 7 μm). This is an advantage for cytogenetic applications that are collectively embraced by the term 'chromosomal genomics' (Doležel et al. 2007). Using synchronised root tips as the source material, chromosome suspensions can be produced as starting material for flow-cytometric sorting of relatively pure fractions of individual Triticeae chromosomes or chromosome arms. The obtained DNA is of high quality (even after mild fixation) and is amenable to enzymatic modifications. This has been demonstrated by preparing chromosome-specific BAC libraries from such source of DNA (Doležel et al. 2007) and by using the DNA to assign SNP loci by PCR to individual barley chromosomes that had been subjected to multiple-strand displacement amplification (MDA) (Šimková et al. 2008). Importantly, all barley chromosomes can be obtained as pure fractions by flow sorting. While the smallest barley chromosome, 1H, can be separated directly from many different cultivars, all other chromosomes need to be purified as separated arms from ditelosomic wheat/barley addition lines (Suchánková et al. 2006). Purified chromosome 1H was MDA amplified, and 800 Mbp of shotgun sequence data was generated by Roche/454 GSFLX sequencing which compares to little more than onefold coverage of this

respective chromosome (Mayer et al. 2009). Almost 80 % of the sequence information was assigned (by Blast and k-mer analysis) to the repetitive DNA fraction of the barley genome. The remaining sequences were then compared to sequence-based genetic markers derived from ESTs (Close et al. 2009), and it was shown that about 90 % of the marker sequences detected had been genetically mapped to chromosome 1H (Mayer et al. 2009). This key result demonstrated that the purified and amplified DNA utilised as template for sequencing originated mainly from the expected chromosome. Hence, the approach demonstrated that it should be possible to assemble an index of sequence tags for possibly all genes represented on a specific barley chromosome. However, it also demonstrated an approach suited to any chromosome or chromosome arm that could be specifically isolated using chromosomal genomics—and established a key component in the strategy to tackle even larger and more complex genomes (e.g. wheat).

As discussed previously, RFLP markers were the first molecular tool that demonstrated that grass genomes exhibit extensive conservation of synteny (Moore et al. 1995). On the basis of the dense gene-based marker maps, an improved model of conserved synteny between barley and other grass genomes has since been established (Thiel et al. 2009). The relationship between rice and sorghum in particular was revisited on the basis of the barley chromosome 1H-specific shotgun sequence information. The dense barley gene-based marker map of this chromosome served as a scaffold to determine all collinear genes between the barley, rice and sorghum genomes. Then, all genes that were not genetically mapped in barley but had been identified amongst the shotgun sequences from specific chromosomes were tested for their collinearity between the sequenced model grasses. Observed collinearity between rice and sorghum was used as a measure to determine the probability that the orthologous genes on barley chromosome 1H would also remain as a conserved syntenic block in the barley genome. On this basis a virtual map ('genome zipper') suggesting the order of over 1,900 barley genes of chromosome 1H was initially developed (Mayer et al. 2009). This study served as a proof of concept, and by relying on the improved Roche/454 GS FLX Titanium technology, producing on average sequence reads longer than 350 nt, all remaining 12 barley chromosome arms were sequenced to over onefold sequence coverage. By including the sequence of *Brachypodium distachyon* (The International Brachypodium Initiative 2010), 'genome zippers' were developed for all barley chromosomes providing access to sequence tags of over 21,000 linearly ordered barley genes (Mayer et al. 2011). This approach was meanwhile also applied to rye (Martis et al. 2013) and wheat chromosomes (Berkman et al. 2011; Hernandez et al. 2011; Wicker et al. 2011; IWGSC 2014) providing completely new opportunities for Triticeae comparative genomics.

14.3.4 Whole Genome Sequencing

14.3.4.1 Whole Genome Shotgun Sequencing

Whole genome shotgun (WGS) sequencing is an efficient strategy to generate draft genome assemblies (Feuillet et al. 2011) and has also been applied to barley. An assembly from Illumina GAIIx/HiSeq short-read sequences providing 50-fold haploid genome coverage was obtained (IBSC 2012). The draft assembly is however not representative of the entire barley genome since only 1.9 Gbp of the 5.1 Gbp genome assembled properly. This unsurprising result illustrates the main limitation of de novo WGS assembly in large and repetitive DNA-rich genomes: the repetitive DNA fails to assemble. However, the practical value of such an assembly is immense, providing direct access to most genes present in a given genome. In barley, 26,159 high-confidence genes (provided with gene expression data support) could be annotated on the contig sequences of the assembly, and since 300 Mbp of WGS contig sequences could be anchored to the physical/genetic map of barley, over 24,000 genes could be integrated to this genomic framework (IBSC 2012). This resource provides completely new possibilities for visualising sequence diversity in a genome-wide context (Fig. 14.1). Re-sequencing information obtained from different cultivars and one accession of wild barley (*Hordeum vulgare* ssp. *spontaneum*) revealed that genetic diversity decreases from the telomeres to the pericentromeric region and the centromeres (IBSC 2012). Even without a true reference genome sequence, on the basis of the sequence-enriched physical/genetic map, it is feasible now to establish sequencing-based mapping and cloning strategies (e.g. Mascher et al. 2014) and to survey the barley genome for patterns of selection imposed during domestication and modern plant breeding, as has recently been demonstrated for other crop species like maize (Hufford et al. 2012; Jiao et al. 2012).

14.3.4.2 Hierarchical Map-Based Sequencing

Although WGS data integrated into a genetic/physical genome framework has proven to be highly enabling for molecular genomic approaches in barley research, the current sequence assembly does not reveal the entire genome information. This limitation is important because, in humans (The ENCODE project consortium 2012) and maize (Chia et al. 2012), the intergenic regions and major contributors to the regulation of gene expression and other processes in the genome (i.e. genomic imprinting, epigenetic changes) are highly transcribed. Unfortunately, we still need to unlock this part of the barley genome—a major goal of current research effort. Once achieved it will allow a better understanding of barley biology and provide understanding of trait selection in plant breeding. Given that access to a true reference sequence of the genome remains an important goal, the IBSC is now working towards this goal by hierarchical clone-by-clone sequencing of the

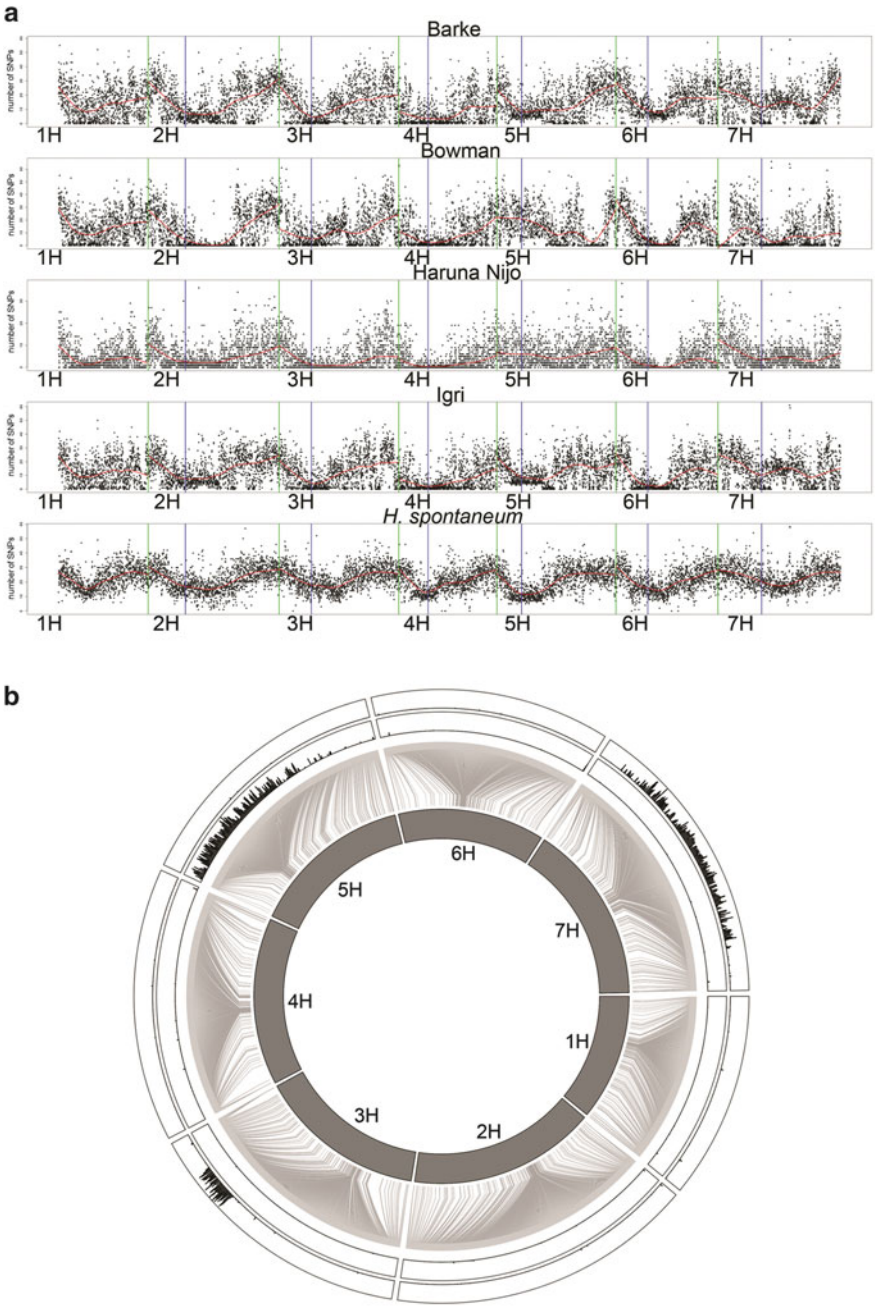

Fig. 14.1 Visualisation of barley sequence diversity. (**a**) Single nucleotide variation (SNV) has been surveyed in whole-genome shotgun (WGS) sequence information of four barley cultivars and one wild barley accession and has been visualised against the sequence-informed physical/genetic map of barley (IBSC 2012). Chromosomes are separated by *green vertical lines*. Centromere positions are indicated by *blue vertical lines*. The median SNV frequency is visualised by a *red line*. Sequence diversity is highest at the telomeric regions of all chromosomes and drops towards

minimum tiling path (MTP) of nonredundant overlapping BAC clones deduced from the physical map (IBSC 2012, Ariyadasa et al. 2014). A project to sequence the entire MTP of chromosome 3H has been initiated in 2010, and by the end of 2012, all 8,863 BAC clones had been draft sequenced (relying on Roche 454 GSFLX and/or Illumina HiSeq 2000 technology) and assembled (Stein et al. unpublished data). MTP sequencing for other chromosomes is currently underway (IBSC members, unpublished data). Indeed, it seems a realistic scenario to expect that all chromosomes of barley will have been draft sequenced by summer of 2014. Sequencing is of course only the first step, and assembly, curation and functional annotation still require a major and most likely international effort; however, initial tools for automated annotation of Triticeae genomic sequences are under development (Leroy et al. 2012).

Conclusions

Over the past decade, genomic research has converted barley from a genetic to a genomic model plant for Triticeae research. The current genome and sequence-based resources will greatly facilitate the cloning of important genes. Ultimately, gene isolation will no longer be more laborious than in rice or other sequenced plant species. Novel sequence-based approaches like sequence-based mapping and cloning are currently at proof of principle stage. In few words—genomic information for barley is no longer a truly limiting factor. Once again, we are returning to the position where successes in barley genetics will be achieved by having the right plant materials in place and the tools to effectively interrogate them. Already the focus has shifted to establishing improved and automated ways of precisely phenotyping traits at all stages of plant development and in as many as possible environments. In fact, high-throughput precision phenotyping has been identified as a major bottleneck (Furbank and Tester 2011; see also Chap. 22) to tackle efficiently quantitatively inherited traits. Combining all these resources will enable breeders to develop new selection schemes, e.g. genomics selection and new opportunities to accelerate barley improvement.

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Fig. 14.1 (continued) the pericentromeric regions. (**b**) WGS data of two nearly isogenic lines carrying known introgressions on chromosomes 3H/7H and 5H, respectively, was obtained and surveyed for SNV (Stein et al. unpublished data). The visualisation of sequence diversity distribution (*black histograms* in outer circles) illustrates the accuracy and resolution that have been obtained by integration of physical and genetic map of barley with extensive sequence information

References

Ariyadasa R, Mascher M, Nussbaumer T, Schulte D, Frenkel Z, Poursarebani N, Zhou R, Steuernagel B, Gundlach H, Taudien S, Felder M, Platzer M, Himmelbach A, Schmutzer T, Hedley P, Muehlbauer G, Scholz U, Korol A, Mayer K, Waugh R, Langridge P, Graner A, Stein N (2014) A sequence-ready physical map of barley anchored genetically by two million SNPs. Plant Physiol 164:412–423

Becker J, Vos P, Kuiper M, Salamini F, Heun M (1995) Combined mapping of AFLP and RFLP markers in barley. Mol Gen Genet 249:65–73

Bennetzen JL, Freeling M (1993) Grasses as a single genetic system: genome composition, collinearity and compatibility. Trends Genet 9:259–261

Bennetzen JL, Freeling M (1997) The unified grass genome: synergy in synteny. Genome Res 7:301–306

Berkman P, Skarshewski A, Manoli S, Lorenc M, Stiller J, Smits L, Lai K, Campbell E, Kubaláková M, Šimková H, Batley J, Doležel J, Hernandez P, Edwards D (2011) Sequencing wheat chromosome arm 7BS delimits the 7BS/4AL translocation and reveals homoeologous gene conservation. Theor Appl Genet 124:423–432

Chia J-M, Song C, Bradbury PJ, Costich D, de Leon N, Doebley J, Elshire RJ, Gaut B, Geller L, Glaubitz JC, Gore M, Guill KE, Holland J, Hufford MB, Lai J, Li M, Liu X, Lu Y, McCombie R, Nelson R, Poland J, Prasanna BM, Pyhajarvi T, Rong T, Sekhon RS, Sun Q, Tenaillon MI, Tian F, Wang J, Xu X, Zhang Z, Kaeppler SM, Ross-Ibarra J, McMullen MD, Buckler ES, Zhang G, Xu Y, Ware D (2012) Maize HapMap2 identifies extant variation from a genome in flux. Nat Genet 44:803–807

Close TJ, Wanamaker SI, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP (2004) A new resource for cereal genomics: 22K barley GeneChip comes of age. Plant Physiol 134:960–968

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney R, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582

Comadran J, Kilian B, Russell J, Ramsay L, Stein N, Ganal M, Shaw P, Bayer M, Thomas W, Marshall D, Hedley P, Tondelli A, Pecchioni N, Francia E, Korzun V, Walther A, Waugh R (2012) A homologue of *Antirrhinum CENTRORADIALIS* is a component of the quantitative photoperiod and vernalization independent *EARLINESS PER SE 2* locus in cultivated barley. Nat Genet 44:1388–1392

Doležel J, Greilhuber J, Lucretti S, Meister A, Lysák MA, Nardi L, Obermayer R (1998) Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann Bot 82:17–26

Doležel J, Kubaláková M, Paux E, Bartoš J, Feuillet C (2007) Chromosome-based genomics in the cereals. Chromosome Res 15:51–66

Druka A, Muehlbauer G, Druka I, Caldo R, Baumann U, Rostoks N, Schreiber A, Wise R, Close T, Kleinhofs A, Graner A, Schulman A, Langridge P, Sato K, Hayes P, McNicol J, Marshall D, Waugh R (2006) An atlas of gene expression from seed to seed through barley development. Funct Integr Genomics 6:202–211

Eversole K, Graner A, Stein N (2009) Wheat and barley genome sequencing. In: Muehlbauer GJ, Feuillet C (eds) Genetics and genomics of the Triticeae. Springer, New York, NY, pp 713–742

Feuillet C, Leach JE, Rogers J, Schnable PS, Eversole K (2011) Crop genome sequencing: lessons and rationales. Trends Plant Sci 16:77–88

Furbank RT, Tester M (2011) Phenomics – technologies to relieve the phenotyping bottleneck. Trends Plant Sci 16:635–644

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Wenzel G, Herrmann RG (1991) Construction of an RFLP map of barley. Theor Appl Genet 83:250–256

Hernandez P, Martis M, Dorado G, Pfeifer M, Gálvez S, Schaaf S, Jouve N, Šimková H, Valárik M, Doležel J, Mayer KFX (2011) Next-generation sequencing and syntenic integration of flow-sorted arms of wheat chromosome 4A exposes the chromosome structure and gene content. Plant J 69:377–386

Hufford MB, Xu X, van Heerwaarden J, Pyhajarvi T, Chia J-M, Cartwright RA, Elshire RJ, Glaubitz JC, Guill KE, Kaeppler SM, Lai J, Morrell PL, Shannon LM, Song C, Springer NM, Swanson-Wagner RA, Tiffin P, Wang J, Zhang G, Doebley J, McMullen MD, Ware D, Buckler ES, Yang S, Ross-Ibarra J (2012) Comparative population genomics of maize domestication and improvement. Nat Genet 44(7):808–811

Jiao Y, Zhao H, Ren L, Song W, Zeng B, Guo J, Wang B, Liu Z, Chen J, Li W, Zhang M, Xie S, Lai J (2012) Genome-wide genetic changes during modern breeding of maize. Nat Genet 44 (7):812–815

Kleinhofs A, Kilian A, Saghai Maroof MA, Biyashev RM, Hayes P, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705

Kurtz S, Narechania A, Stein J, Ware D (2008) A new method to compute K-mer frequencies and its application to annotate large repetitive plant genomes. BMC Genomics 9:517

Leroy P, Guilhot N, Sakai H, Bernard A, Choulet F, Theil S, Reboux S, Amano N, Flutre T, Pelegrin C, Ohyanagi H, Seidel M, Giacomoni F, Reichstadt M, Alaux M, Gicquello E, Legeai F, Cerutti L, Numa H, Tanaka T, Mayer K, Itoh T, Quesneville H, Feuillet C (2012) TriAnnot: a versatile and high performance pipeline for the automated annotation of plant genomes. Front Plant Sci 3:5

Lonardi S, Duma D, Alpert M, Cordero F, Beccuti M, Bhat PR, YonghuiWu, Ciardo G, Alsaihati B, Ma Y, Wanamaker S, Resnik J, Close TJ (2012) Barcoding-free BAC pooling enables combinatorial selective sequencing of the barley gene space. arXiv:11124438v1 [q-bioGN]

Mardis ER (2008) The impact of next-generation sequencing technology on genetics. Trends Genet 24:133–141

Martis MM, Zhou R, Haseneyer G, Schmutzer T, Vrána J, Kubaláková M, König S, Kugler KG, Scholz U, Hackauf B, Korzun V, Schön C-C, Doležel J, Bauer E, Mayer KFX, Stein N (2013) Reticulate evolution of the rye genome. Plant Cell 25:3685–3698

Mascher M, Jost M, Kuon J-E, Himmelbach A, Aßfalg A, Beier S, Scholz U, Graner A, Stein N (2014) Mapping-by-sequencing accelerates forward genetics in barley. Genome Biol 15:R78

Matsumoto T, Tanaka T, Sakai H, Amano N, Kanamori H, Kurita K, Kikuta A, Kamiya K, Yamamoto M, Ikawa H, Fujii N, Hori K, Itoh T, Sato K (2011) Comprehensive sequence analysis of 24,783 barley full-length cDNAs derived from 12 clone libraries. Plant Physiol 156:20–28

Mayer KFX, Taudien S, Martis M, Simkova H, Suchankova P, Gundlach H, Wicker T, Petzold A, Felder M, Steuernagel B, Scholz U, Graner A, Platzer M, Dolezel J, Stein N (2009) Gene content and virtual gene order of barley chromosome 1H. Plant Physiol 151:496–505

Mayer KFX, Martis M, Hedley P, Šimková H, Liu H, Morris JA, Steuernagel B, Taudien S, Roessner S, Gundlach H, Kubaláková M, Suchánková P, Murat F, Felder M, Nussbaumer T, Graner A, Salse J, Endo T, Sakai H, Tanaka T, Itoh T, Sato K, Platzer M, Matsumoto T, Scholz U, Doležel J, Waugh R, Stein N (2011) Unlocking the barley genome by chromosomal and comparative genomics. Plant Cell 23:1249–1263

Michalek W, Künzel G, Graner A (1999) Sequence analysis and gene identification in a set of mapped RFLP markers in barley (Hordeum vulgare). Genome 42:849–853

Moore G, Devos KM, Wang Z, Gale MD (1995) Grasses, line up and form a circle. Curr Biol 5:737–739

Muñoz-Amatriaín M, Moscou MJ, Bhat PR, Svensson JT, Bartoš J, Suchánková P, Šimková H, Endo TR, Fenton RD, Lonardi S, Castillo AM, Chao S, Cistué L, Cuesta-Marcos A, Forrest KL, Hayden MJ, Hayes PM, Horsley RD, Makoto K, Moody D, Sato K, Vallés MP, Wulff BBH, Muehlbauer GJ, Doležel J, Close TJ (2011) An improved consensus linkage map of barley based on flow-sorted chromosomes and single nucleotide polymorphism markers. Plant Genome 4:238–249

Paux E, Roger D, Badaeva E, Gay G, Bernard M, Sourdille P, Feuillet C (2006) Characterizing the composition and evolution of homoeologous genomes in hexaploid wheat through BAC-end sequencing on chromosome 3B. Plant J 48:463–474

Potokina E, Druka A, Luo Z, Wise R, Waugh R, Kearsey M (2008) Gene expression quantitative trait locus analysis of 16,000 barley genes reveals a complex pattern of genome-wide transcriptional regulation. Plant J 53:90–101

Qi X, Stam P, Lindhout P (1998) Use of locus-specific AFLP markers to construct a high-density molecular map in barley. Theor Appl Genet 96:376

Ramsay L, Macaulay M, Cardle L, Morgante M, Sd I, Maestri E, Powell W, Waugh R (1999) Intimate association of microsatellite repeats with retrotransposons and other dispersed repetitive elements in barley. Plant J 17:415–425

Sato K, Nankaku N, Takeda K (2009) A high-density transcript linkage map of barley derived from a single population. Heredity 103:110–117

Sato K, Motoi Y, Yamaji N, Yoshida H (2011) 454 sequencing of pooled BAC clones on chromosome 3H of barley. BMC Genomics 12:246

Schulte D, Close TJ, Graner A, Langridge P, Matsumoto T, Muehlbauer G, Sato K, Schulman AH, Waugh R, Wise RP, Stein N (2009) The international barley sequencing consortium–at the threshold of efficient access to the barley genome. Plant Physiol 149:142–147

Service R (2006) The race for the $1000 genome. Science 311:1544–1546

Šimková H, Svensson JT, Condamine P, Hřibová E, Suchánková P, Bhat PR, Bartoš J, Šafář J, Close TJ, Doležel J (2008) Coupling amplified DNA from flow-sorted chromosomes to high-density SNP mapping in barley. BMC Genomics 9:294

Sreenivasulu N, Usadel B, Winter A, Radchuk V, Scholz U, Stein N, Weschke W, Strickert M, Close TJ, Stitt M, Graner A, Wobus U (2008) Barley grain maturation and germination: metabolic pathway and regulatory network commonalities and differences highlighted by new MapMan/PageMan profiling tools. Plant Physiol 146:1738–1758

Stein N (2007) Triticeae genomics: advances in sequence analysis of large genome cereal crops. Chromosome Res 15:21–31

Stein N, Prasad M, Scholz U, Thiel T, Zhang H, Wolf M, Kota R, Varshney RK, Perovic D, Graner A (2007) A 1000 loci transcript map of the barley genome – new anchoring points for integrative grass genomics. Theor Appl Genet 114:823–839

Steuernagel B, Taudien S, Gundlach H, Seidel M, Wicker T, Ariyadasa R, Schulte D, Petzold A, Felder M, Graner A, Scholz U, Mayer K, Platzer M, Stein N (2009) De novo 454 sequencing of barcoded BAC pools for comprehensive gene survey and genome analysis in the complex genome of barley. BMC Genomics 10:547

Suchánková P, Kubaláková M, Kovářová P, Bartoš J, Číhalíková J, Molnár-Láng M, Endo TR, Doležel J (2006) Dissection of the nuclear genome of barley by chromosome flow sorting. Theor Appl Genet 113:651–659

Svensson JT, Crosatti C, Campoli C, Bassi R, Stanca AM, Close TJ, Cattivelli L (2006) Transcriptome analysis of cold acclimation in barley Albina and Xantha mutants. Plant Physiol 141:257–270

Taudien S, Steuernagel B, Ariyadasa R, Schulte D, Schmutzer T, Groth M, Felder M, Petzold A, Scholz U, Mayer KF, Stein N, Platzer M (2011) Sequencing of BAC pools by different next generation sequencing platforms and strategies. BMC Res Notes 4:411

The ENCODE project consortium (2012) An integrated encyclopedia of DNA elements in the human genome. Nature 489:57–74

The International Barley Genome Sequencing Consortium (IBSC) (2012) A physical, genetical and functional sequence assembly of the barley genome. Nature 491:711–716

The International Brachypodium Initiative (2010) Genome sequencing and analysis of the model grass Brachypodium distachyon. Nature 463:763–768

The International Wheat Genome Sequencing Consortium (2014) A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. Science 345. doi:10.1126/science.1251788

Thiel T, Michalek W, Varshney R, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (Hordeum vulgare L.). Theor Appl Genet 106:411–422

Thiel T, Graner A, Waugh R, Grosse I, Close TJ, Stein N (2009) Evidence and evolutionary analysis of ancient whole-genome duplication in barley predating the divergence from rice. BMC Evol Biol 9:209

Varshney RK, Marcel TC, Ramsay L, Russell J, Röder MS, Stein N, Waugh R, Langridge P, Niks RE, Graner A (2007) A high density barley microsatellite consensus map with 775 SSR loci. Theor Appl Genet 114:1091–1116

Wicker T, Schlagenhauf E, Graner A, Close TJ, Keller B, Stein N (2006) 454 sequencing put to the test using the complex genome of barley. BMC Genomics 7:275

Wicker T, Narechania A, Sabot F, Stein J, Vu GTH, Graner A, Ware D, Stein N (2008) Low-pass shotgun sequencing of the barley genome facilitates rapid identification of genes, conserved non-coding sequences and novel repeats. BMC Genomics 9:518

Wicker T, Taudien S, Houben A, Keller B, Graner A, Platzer M, Stein N (2009) A whole-genome snapshot of 454 sequences exposes the composition of the barley genome and provides evidence for parallel evolution of genome size in wheat and barley. Plant J 59:712–722

Wicker T, Mayer KFX, Gundlach H, Steuernagel B, Scholz U, Šimková H, Kubaláková M, Choulet F, Taudien S, Platzer M, Feuillet C, Fahima T, Budak H, Keller B, Doležel J, Stein N (2011) Frequent gene movement and pseudogene evolution is common to the large and complex genomes of wheat, barley, and their relatives. Plant Cell 23:1706–1718

Wise R, Caldo R, Hong L, Shen L, Cannon E, Dickerson J (2007a) BarleyBase/PLEXdb: a unified expression profiling database for plants and plant pathogens. In: Edwards D (ed) Methods in molecular biology, plant bioinformatics – methods and protocols. Humana, Totowa, NJ, pp 347–363

Wise R, Moscou M, Bogdanove A, Whitham SA (2007b) Transcript profiling in host-pathogen interactions. Annu Rev Phytopathol 45:329–369

Zhang H, Sreenivasulu N, Weschke W, Stein N, Rudd S, Radchuk V, Potokina E, Scholz U, Schweizer P, Zierold U, Langridge P, Varshney RK, Wobus U, Graner A (2004) Large-scale analysis of the barley transcriptome based on expressed sequence tags. Plant J 40:276–290

Chapter 15
Induced Genetic Variation, TILLING and NGS-Based Cloning

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15.1 Introduction

Mutagenesis is one of the most important tools available to barley geneticists and breeders in order to investigate trait inheritance and to provide useful genetic variation to breeding programmes. Recent advancements in genomics, including the increasing availability of barley genome sequence information, are making mutagenesis even more valuable. In a forward genetics perspective (from traits to genes), the main improvements are being obtained by the exploitation of high-throughput phenotyping and genotyping. SNP genotyping and next-generation sequencing (NGS) platforms enable to genetically and physically map, or even to clone, target mutant genes in single-step experiments, once segregating populations are available. In barley, reverse genetics (from genes to traits), both transposon-based mutagenised populations and multiple TILLING resources, are becoming available or increasing their coverage. These resources too can be made more effective if matched with NGS-based molecular screening.

One of the most prominent achievements in the history of genetics was the discovery of mutation induction by applying physical and chemical agents (Kharkwal 2012). The research was pioneered by two American geneticists, H. J. Muller (1927) and L. J. Stadler (1928, 1930), the latter already using barley as a model system. Soon after these reports, research on barley mutagenesis began,

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including an important and long-lived programme led by H. Nilsson-Ehle and A. Gustafsson at the Svalöv AB Laboratory, Sweden (Lundqvist 2009). Barley was chosen as one of the main models in research on mutation induction mainly because it is a diploid self-fertilising species that rarely outcrosses and produces a sufficiently large progeny from a single plant. Since then, barley mutagenesis has contributed strongly to basic plant biology (see, for instance, Forster et al. 2007) and also contributed to the release of more than 270 mutant varieties (IAEA/FAO Mutant Varieties Database at www.mvd.iaea.org/MVD/default.htm), therefore improving agriculture and food security worldwide (Kharkwal and Shu 2009). Today, physical and chemical mutagenesis is still being applied most widely both in 'forward' (see Sect. 15.4) and 'reverse' genetics (see Sect. 15.5.1) fashions, despite the recent progress in the development of systems aimed at determining the gene function on the basis of insertional mutagenesis or other novel approaches (see Sects. 15.5.2 and 15.5.3).

15.2 Chemical and Physical Mutagenesis

For over 80 years of mutation induction in the barley genome, various radiation types (physical mutagens) have been applied. The mutagenic agents have included X-rays, γ-rays (acute and chronic, produced by radioisotopes of ^{60}Co and ^{137}Cs), neutrons (fast and thermal), electrons, protons, α-rays from radon and β-rays from ^{32}P and ^{35}S and, recently, ion beam (Table 15.1) (van Harten 1998; Mba et al. 2012). These types of ionising radiation vary in the density of the ions produced and in the distribution of the ionising rays and can be categorised as follows: sparsely ionising radiation (X-rays, γ-rays) and densely ionising radiation (fast and thermal neutron, electrons, protons as well as α-rays and β-rays). These types of electromagnetic radiation are emitted as quanta but differ in wavelengths and particular characteristics. Each type of ionising radiation generates deletions at a high frequency, which are usually larger with fast-neutron irradiation. Deletions are valuable for many experiments because they often result in the loss of gene function and are often recognisable with Southern blot, PCR analysis or more advanced genomic approaches [e.g. deletion mapping, Bruce et al. (2009)]. The doses of physical mutagens which are routinely used for barley seed treatment are given in Table 15.1.

The physical mutagenesis work carried out at the Svalöv Laboratory led to important conclusions: barley seeds are 20–30 times more sensitive to neutron irradiation than to the application of X-rays (when equal doses are used), and the difference in efficiency between irradiation with these two agents results from the difference in the density of the ions produced. Germinating barley seeds are about three times more sensitive to neutrons than dormant ones, mainly due to different water content levels. Neutrons are about ten times more efficient than X-rays of equivalent energy dissipation in producing chromosome disturbances and even 100 times as effective in producing first-generation sterility and increasing the

Table 15.1 Doses of physical and chemical mutagens routinely used for barley seed treatment (Maluszynski et al. 2003, modified)

Type of mutagen	Mutagen		Range of doses
Physical	Fast neutrons	Ionising radiation	2–5 Gy
	Thermal neutrons	Ionising radiation	4×10^7–6.5×10^7 N cm^{-2}
	X-rays	Ionising radiation	60–200 Gy
	Gamma rays	Ionising radiation	150–400 Gy
Chemical	Carbon $^{12}C^{5+}$ ions	Ion beams	220 MeV
	N-methyl-*N*-nitrosourea (MNU)	Alkylating agent	0.5–1.0 mmol l^{-1} × 5 h
	N-ethyl-*N*-nitrosourea (ENU)	Alkylating agent	1.0–2.5 mmol l^{-1} × 5 h
	Ethyl methanesulfonate (EMS)	Alkylating agent	0.02–2.5 % × 8–20 h
	Sodium azide	Mutagenic through an organic moiety (β-azidoalanine)	0.5–1.5 mmol l^{-1} × 5 h

mutation frequency in the second generation. It was also concluded that the number of mutants increases correspondingly to the irradiation doses within the low and medium range, but declines considerably at high dosages (Lundqvist 1992), and it became clear that radiosensitivity tests should be conducted in order to determine effective dose treatment [a description of the methodology is available in Kodym et al. (2012)]. In more recent studies, it has been shown that ion beams, including proton, helium and the heavier charged particles (in barley the carbon $^{12}C^{5+}$ ions at the dose of 220 MeV are most commonly used), are highly mutagenic. In this type of mutagenesis, positively charged ions are accelerated at a high speed (around 20–80 % of the speed of light) and used to irradiate target cells (Magori et al. 2010; Abe et al. 2012a). These mutagenic agents are highly efficient in mutation induction when compared to low-energy transfer radiations, such as gamma rays, X-rays and electrons. Ion beams deposit the high energy on a local target and therefore induce predominantly single- and double-strand breaks with damaged end groups, which are difficult to repair when compared to DNA damage caused by gamma rays and electrons (Tanaka et al. 2010). These damages end mainly as large DNA alterations, namely deletions, inversions and translocations, although a high frequency of point mutations has also been observed in some species (Abe et al. 2012a).

With respect to plant mutation breeding, over 60 % of the mutant varieties in over 200 species listed in the IAEA/FAO mutant variety genetic stock database (http://mvgs.iaea.org) were produced by gamma irradiation. However, gamma emitters involve radioactive isotopes of cobalt and caesium, and there are now stringent regulations on the transport and setup of gamma sources. As a consequence X-ray irradiation protocols are being developed for mutation breeding (BP Forster, pers. comm.). X-ray machines have the advantage of being safer, do

not involve radioactivity, have no toxic chemicals and are readily available (Mba et al. 2012).

As to chemical mutagenesis, since the 1940s a range of chemical compounds, including epoxides and epimines, alkylating and oxidising agents, purine derivatives and alkanesulfonic esters, have been tested in plants [reviewed in Leitao (2012)] including barley. Rather than large deletions, chemical mutagens generate an allelic series at any target locus which may result in a reduction in activity, specificity or loss of the protein function. These types of changes can be very valuable for the assigning of the gene function. The doses of chemical mutagens which are most often used for barley seed treatment are given in Table 15.1. It should be kept in mind that the mutagenic action of a chemical compound in barley may be highly variety dependent as was shown for MNU (Maluszynski et al. 2003). Most of the chemical mutagens cause a specific type of alteration in the DNA sequence. It was shown that EMS produces mostly GC → AT transitions. Similarly, MNU induces mainly GC → AT transitions; however, inversions and translocations were also generated at low frequencies (Szarejko and Maluszynski 1980; Maluszynski et al. 2003). *N*-ethyl-*N*-nitrosourea (ENU) causes both GC → AT (in the majority) and AT → GC transitions, as well as transversions (GC → CG and AT → CG) (Wienholds et al. 2003). Another alkylating agent, diethyl sulfate, proved to be highly mutagenic in barley. It produces mutation rates up to 10 %, twice as high as those obtained by sparsely ionising radiation (Lundqvist 1992). Sodium azide generates mostly AT → GC transitions and is marginally effective in the model species *Arabidopsis thaliana* [Koornneef (2002), Talamè et al. (2008); for a review see Gruszka et al. (2012)]. The high frequency of mutations induced by this chemical, the negligible occurrence of chromosome aberrations, and the low toxicity for human health (Kleinhofs et al. 1978) make sodium azide a particularly efficient mutagen that is very useful for practical barley breeding purposes (Lundqvist 1992). The base analogues 5-bromouracil, 2-aminopurine, 8-ethoxycaffeine and maleic hydrazide show mutagenic activity as well; however, they are rarely used for mutagenesis in plants (Maluszynski et al. 2003). Small deletions (1–2 bp) can be induced by the chemical diepoxybutane (DEB); however, its mechanism of action is still unknown (Caldwell et al. 2004; Waugh et al. 2006).

15.3 Classification of Mutants and Impact on Breeding

A large number of barley morphological mutations have been observed and described (>10,000 mutants for the Swedish mutation research programme alone) (Lundqvist 2009), covering a broad variation range. Comprehensive lists and description are available at the Barley Genetics Stocks AceDB Database (http://ace.untamo.net) and at the GrainGenes database (http://wheat.pw.usda.gov/GG2/index.shtml). Further information is provided in the Barley Genetics Newsletter (Volume 26, 1997, is a special issue reporting description of genetic stocks and mutants) (Lundqvist et al. 1997; Forster et al. 2012). In addition, some of the barley

TILLING populations have been phenotyped and the phenotypes recorded and made available (e.g. TILLMore website at www.dista.unibo.it/TILLMore/index.php).

Barley mutants have been classified using a system of hierarchical categories. Lundqvist (2005, 2009) listed 10 main categories (spike/spikelets, culm length/composition, growth type, kernel development/formation, physiological, awn, leaf blade, pigmentation, chlorophyll, resistance to powdery mildew) with 116 different subtypes. The top represented subtypes are reported in Table 15.2. Similarly, in the phenotypic analysis of 21,000 mutated families from the cv Optic, Forster et al. (2012) utilised a system with 9 main classes and a total of 204 classes. Three mutant categories, namely, late flowering, light green and short stature greatly outnumbered the others. Root mutants have clearly been recognised as underrepresented in collections, although efforts towards their description have been undertaken (White et al. 2009; Bovina et al. 2011a; Forster et al. 2012). Along with mutant description, nomenclature and gene symbols have been refined and standardised (Lundqvist et al. 1997; Franckowiak and Lundqvist 2009).

One major obstacle in the comparative evaluation of mutants is their origin from different lines. To address this obstacle in barley genetics, a large backcross programme was initiated in the mid-1980s to introgress mutations (more than 800 stocks) in a homogeneous genetic background (the two-rowed spring-type cv

Table 15.2 Frequency distribution of barley lines across the 22 top phenotypic groups available at the Nordgen Gene Bank (http://www.nordgen.org/index.php/en/content/view/full/344)

Mutant group	Frequency
Eceriferum	1,871
Double mutant intermedium	1,487
Erectoides	1,203
Praematurum	1,203
Breviaristatum	489
Laxatum	359
Globosum	227
Mildew resistance	222
Irregular spikes	161
Erect growth habit	159
Intermedium spike	129
Maculosus	117
Short culm	108
Seminudoides	91
Curly awns	81
Long-shaped grain	80
Necroticans	79
Narrow leaf blade	72
Light green	71
Macrolepis	61
Short spike	61
Gigas	55

Bowman, used as recurrent parent in the backcross programme) (Druka et al. 2011). The 'Bowman' near-isogenic lines have also been extensively marker genotyped, which provided information on isogenicity and approximate map position of mutants (Druka et al. 2011).

Barley mutants continue to play a key role in plant breeding worldwide. Examples include the development of high-yielding and short-stature barley mutant cvs 'Diamant' and 'Golden Promise', which have made a significant impact on European agriculture and have also been used as the parents of many leading barley cvs in Europe, North America, Asia and Australia. The X-ray-induced cv 'Diamant' was shorter than the parent cv 'Valticky' and had a 12 % yield increase (Petr et al. 2002). The semi-dwarf phenotype of this cv is determined by mutation at *denso* (*sdw1*) locus. Another novel allele of this gene was induced by X-rays in the six-rowed barley cv 'Jotun' and has been widely used for development of semi-dwarf feed barley varieties in the USA, Canada and Australia. The other cv 'Golden Promise' is characterised by stiff straw, high yield and improved malting quality and was produced by γ-ray irradiation of the malting cv 'Maythorpe'. The semi-dwarf phenotype of 'Golden Promise' is determined by the mutation in *ari-e* locus. Recent studies have shown that 'Golden Promise' is also salt tolerant (Forster 2001). A 'suppressor screen' approach (mutagenesis is applied to a line carrying a severe mutation, and selection screening is targeted in order to recover new mutants with altered, usually milder, expression of the original trait) was also recently applied on severe dwarfing barley lines (Chandler and Harding 2013). This led to the identification of 'overgrowth' suppressor mutants of potential breeding interest.

Mutation breeding enabled to extend the environmental range of barley cultivation. The cv 'Mari' was the first induced [by X-ray, from the cv 'Bonus'; Dormling et al. (1966)] early barley mutant to be released in 1961. Remarkably, since its release, 'Mari' or its derivatives have been used worldwide to breed for short-season adaptation. The gene responsible for this early phenotype has now been cloned and shown to correspond to the circadian clock regulator *Elf3* (Zakhrabekova et al. 2012). Early flowering mutants (e.g. cv 'UNA La Molina 95', obtained by γ-ray treatment of 'Buenavista') (Romero Loli and Gomez Pando 2001) also contributed to adaptation to high altitudes (over 3,500 m asl in Peru). Barley mutants contributed to expand the range of cultivation also by providing new source of resistance to pathogens (e.g. *mlo9* mutation for resistance to mildew from cv 'Diamant' and first released in the successful cv 'Alexis' in 1986) (list of *mlo* mutants, *mlo* cultivars and related information is maintained at www.crpmb.org/mlo/index.html).

15.4 Forward Genetics and Next-Generation Sequencing-Enhanced Cloning of Mutants

Cloning genes responsible for phenotypic mutants in barley is not a simple task mainly due to the large (5.1 Gb; Dolezel et al. 1998) and highly repetitive (>80 %; Sreenivasulu et al. 2008) genome. Of the extensive legacy of publicly available genetic resources, including induced mutant and spontaneous off-type collections, wild barleys, landraces and cultivars, only a handful of genes causal to some of these phenotypes have been cloned, leaving many interesting and potentially useful genes yet to be cloned. In this section, we will outline phenotype-based gene cloning in barley emphasising the role of parallel high-throughput genotyping and next-generation sequencing (NGS) technologies to shortcut the process. The diagram in Fig. 15.1 shows the workflow and relationships between different approaches and genetic resources commonly used in these cloning projects. A very important tool in the hands of scientists working in this field is the recently released extensive barley genome sequence assembly which had 3.9 Gb (75 % of the genome) anchored to a high-resolution genetic map, including an estimated >90 % of the genes (IBGSC 2012). This resource along with the increasingly detailed description of synteny across model and crop species with more complete genome sequences (e.g. *Brachypodium* and rice) (Mayer et al. 2011) will strongly positively impact barley mutant cloning.

15.4.1 Steps for Mapping and Cloning a Mutant

The first step in cloning the gene responsible for a mutation is to ensure to have fixed it in a completely homozygous line. It is also convenient to acquire as much information as possible (e.g. mapping position of mutated loci) from previous research work on similar phenotypes. Additionally, complementation tests between the target mutant and other described mutants with similar phenotypes should be carried out. For this purpose, seeds of lines carrying potentially alternative alleles can be requested from existing stock repositories or can be obtained by designing and performing new mutagenesis experiments. Examples of publicly accessible barley seed depositories are the Nordic Genetic Resource Center or NordGen, Alnarp, Sweden (http://www.nordgen.org/); National Small Grains Collection (NSGC), Aberdeen, Idaho, USA (www.ars.usda.gov/Main/docs.htm?docid=21891); Barley and Wild Plant Resource Center, Okayama University, Japan (www.shigen.nig.ac.jp/barley/) and John Innes Centre Germplasm Resources Unit (GRU), Norwich, UK (www.jic.ac.uk/germplas/). For many of barley phenotypes, allele/gene relationships have already been determined (Lundqvist et al. 1997; see also the Barley Genetics Stocks AceDB) and their map position experimentally verified and integrated in consensus maps. For example, the Oregon Wolfe Barley doubled haploid population (Costa et al. 2001) segregates at

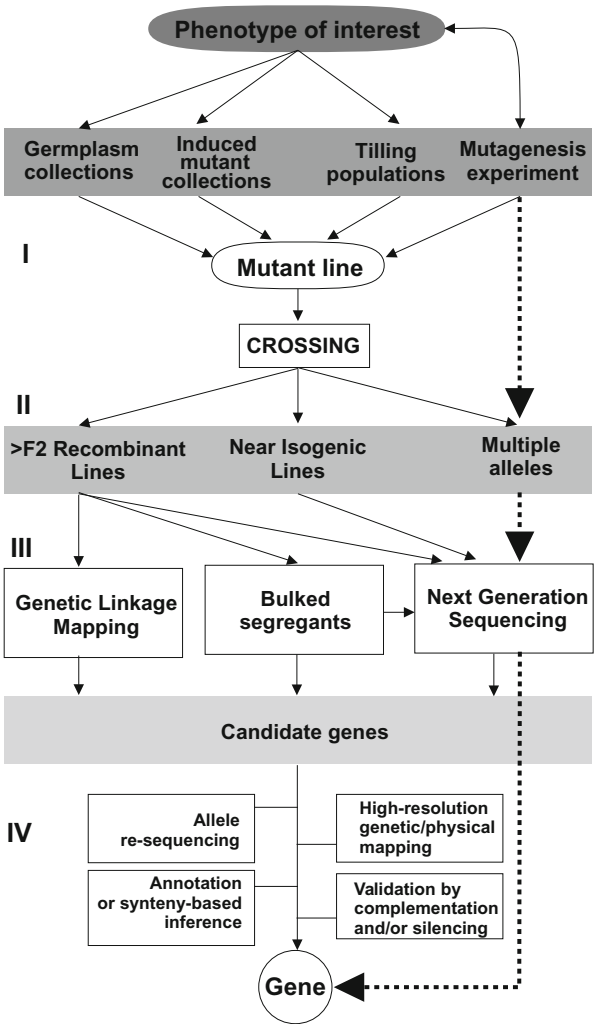

Fig. 15.1 Phenotype-based cloning of barley genes. The key steps (I–IV), resources and approaches are shown. The *dotted line* indicates one of the paths enabled by NGS which may not require crossing and population development (Schneeberger and Weigel 2011)

12 morphological loci including loci controlling the following traits: six-rowed spike1 (*Vrn1*), black lemma and pericarp (*Blp*), hairy sheath (*Hsh*) and others. Similarly, Pozzi et al. (2003) integrated 29 developmental mutants into a molecular marker linkage map.

The second step is to cross the identified lines in order to (1) generate a mapping population and (2) clean and/or replace the mutated background genome (by backcrossing).

The third step includes phenotyping and genotyping of the populations followed by linkage mapping of the phenotype. Since its first report in barley (Rostoks et al. 2006), BeadArray SNP detection platform based on Golden Gate assays (Illumina Inc., CA, USA) has been used in a number of genetic studies including

development of the reference barley genetic linkage map (Close et al. 2009), association mapping studies (Ramsay et al. 2011) and mapping of wild barley introgression lines (von Korpf et al. 2010). The technology exploits known SNP-spanning sequences based on which oligo pool arrays (OPAs)—typically containing 1,536 SNP assays—are designed. The output of this type of assay is represented by two numerical values (X and Y) for each SNP in each DNA sample (Fig. 15.2). Recently, based on a similar technology (Infinium Illumina), a format including approximately 8,000 barley SNPs has also been made available (Comadran et al. 2012). A number of statistical tools have been developed in

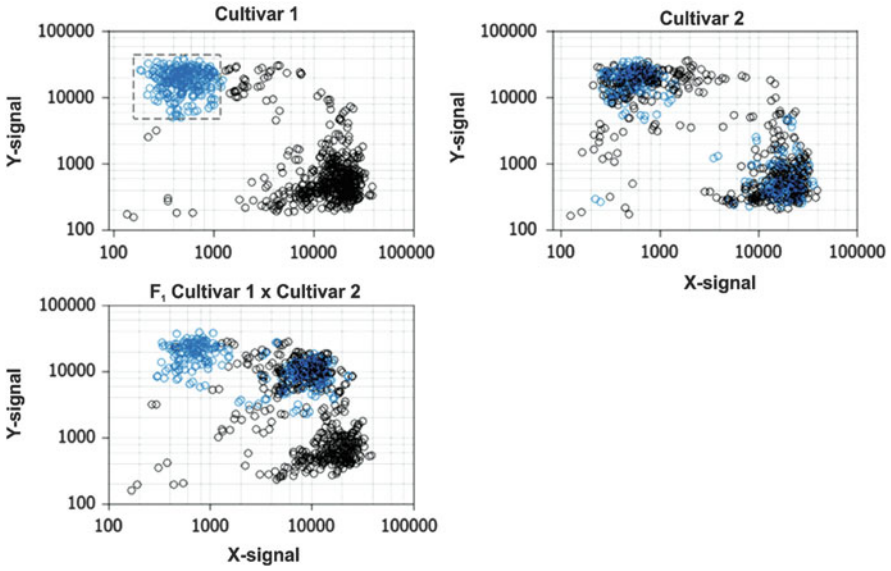

Fig. 15.2 Example of graphical representation of a SNP-based genotyping array output (Illumina BeadArray). The output refers to the analysis of two barley cultivars and their F_1 hybrid. In this type of assay, each SNP genotype call in each DNA sample is associated to two numerical values (X- and Y-signal). For homozygous loci, one of these values is high and the other low, whereas for heterozygous loci both values are similarly high. The genotyping output of a single barley cultivar ('Cultivar 1' or 'Cultivar 2') typically results in two major clusters of SNP-associate point values. The two major clusters represent most of the SNPs, and ideally they should divide about equally between the low and high values. For example, one of the highlighted SNP groups includes 205 SNPs or 53 % of all SNPs on a 384 SNP array. SNPs that fall between the '*upper-left*' and '*lower-right*' clusters may represent some residual heterozygous SNP or non-specific signals. SNPs that both have low values are either failed assays or may indicate the presence of insertion/deletion type of polymorphism. The scatter plot shown in 'Cultivar 2' shows distribution of the SNP signals in a different cultivar highlighting the same subset of SNPs as in 'Cultivar 1'. In 'Cultivar 2', not all highlighted SNPs are in the '*upper-left*' cluster, rather they are distributed between both major clusters. Those that moved outside the cluster highlighted in 'Cultivar 1' are polymorphic between both cultivars. The third panel shows distribution of SNP values in the F_1 hybrid genome made by crossing 'Cultivar 1' with 'Cultivar 2'. In between the two expected clusters X and Y, a third major cluster has appeared. This cluster represents heterozygous SNPs and hence polymorphic SNPs between the two cultivars

order to standardise the raw data and score SNP genotypes as accurately as possible (Li et al. 2012; Teo 2012). The effectiveness of these technologies is due to the fact that hundreds or thousands of DNA samples can easily be analysed in parallel. This allows generating a de novo linkage map for the whole genome in one experiment. SNP arrays can also be used in bulked-segregant analysis (BSA) (Michelmore et al. 1991), where DNA from 8 to 10 recombinant lines with the same phenotype is pooled together and then genotyped. Depending on how representative the genotyping platform is, BSA can identify closely linked markers and/or gene of interest itself (Trick et al. 2012). BSA can also be combined with NGS technologies (see Sect. 15.4.2).

A different mapping approach involves genotyping and comparing backcross-derived lines (which show a target phenotype) with their recurrent nearly isogenic parent. Polymorphic SNPs from the same chromosomal region can be identified which will roughly indicate the map position of the target gene. This approach has been applied using a 1,536 SNP arrays to a collection of over 800 barley backcross-derived lines that were generated using the cv 'Bowman' as recurrent parent (Druka et al. 2011).

The fourth and the last step in the cloning process is the identification of the candidate genes and, among them, the one causally responsible for the phenotype. An important support is provided by the genome synteny between barley and other phylogenetically related species (wheat, rice, maize, sorghum or *Brachypodium*) holding a fully sequenced genome. One of the most comprehensive tools that exploit synteny and genomic information is the Genome Zipper (Mayer et al. 2011). In one platform, it combines data from flow-sorted chromosome arm sequencing (Šimková et al. 2008), barley reference linkage map (Close et al. 2009) and sequenced grass genomes.

Once a list of candidate genes is made available several options are possible. If multiple allelic variants for the phenotype of interest exist, the most straightforward approach is to re-sequence the candidate genes from them and from original parental line that was used for mutagenesis. The gene with different non-conservative mutations in different allelic variants is very likely causal to the phenotype of interest. This information has already proved to be useful in phenotype-based gene cloning projects (Komatsuda et al. 2007; Ramsay et al. 2011; Houston et al. 2012; Zakhrabekova et al. 2012).

However, there are many interesting and potentially useful genes that have only two known alternative alleles (wild type and mutant). In this case, two options exist: (1) fine mapping of the phenotype (by means of large segregating populations) combined with physical mapping (e.g. based on BAC library), the exploitation of the assembled genome sequence (IBGSC 2012) and synteny information (e.g. Mayer et al. 2011), in order to define a genetic region small enough to contain one gene co-segregating with the phenotype; or (2) if few candidate genes emerged from genetic mapping, a functional analysis by deploying one or more reverse genetics tools (see Sect. 15.5) should be applied.

15.4.2 Applications of NGS Technologies to Forward Genetics

NGS technologies [for reviews see Blaby-Haas and de Crécy-Lagard (2011), Jackson et al. (2011)] may considerably shortcut the forward genetics cloning process. Depending on the context, NGS can be applied to different steps of the process (Fig. 15.1). Low-pass genome NGS and genotype by sequencing (GBS) enable to quickly reveal and map thousands of markers in mapping populations (Elshire et al. 2011; van Oeveren et al. 2011; Wang et al. 2011). QTL-NILs (and of course mutant NILs) can be quickly and exhaustively characterised (Earley and Jones 2011). Mapping approaches combining NGS with BSA [ShoreMap and MutMap. Schneeberger et al. (2009) and Abe et al. (2012b)] appear particularly powerful. In these approaches, the two BSA bulks (containing DNA of either mutated or WT plants from a segregating population) are deep-sequenced using NGS, rather than genotyped as in original BSA. Thanks to the very high read coverage provided by NGS, virtually all sequence polymorphisms and recombinant chromosomes present in the bulks are screened and utilised for mapping in one-step experiment. Therefore, in a situation where a mapping population is available and phenotyped, such NGS-enhanced BSA allows a single investigator to identify the causative mutation underlying a mutant in only a few days of work, which is probably at least 10 times faster than traditional methods (Schneeberger and Weigel 2011). As expected, this approach is rapidly being adopted by plant geneticists (Schneeberger et al. 2009; Cuperus et al. 2010; Uchida et al. 2011; Trick et al. 2012). Unfortunately, the dimension of the barley genome is such that BSA combined with whole-genome NGS is not yet affordable. However, NGS-BSA can already be applied if a genome complexity reduction step is introduced prior to genome sequencing. One approach to obtain such reduction is by exome sequencing (Bamshad et al. 2011). Exome sequencing is based on NGS re-sequencing of whole-genome gene space which is captured by means of oligonucleotide probes. A barley-specific exome-capture array encompassing >60 Mb nonredundant coding sequences has been developed by the members of the International Barley Genome Sequencing Consortium (Mascher et al. 2013), and it has already been utilized to clone two barley mutants (Mascher et al. 2014; Pankin et al. 2014).

15.5 Reverse Genetics

In reverse genetics, the pre-existing information about the molecular nature and structure of a gene is exploited in order to investigate, test or validate its function. This is obtained by acting on the structure of a gene by mutagenesis, followed by the recovery of the individuals carrying the mutation, which are then phenotyped. Strategies for reverse genetics that are currently important in barley genetics include TILLING (see Sect. 15.5.1) and insertional mutagenesis by transposons

or T-DNA insertion (see Sect. 15.5.2). It can also be easily predicted that strategies involving gene targeting and gene substitution, now in their infancy, will also find important applications in barley (see Sect. 15.5.3). Although not treated here, the investigation of gene function at the genomic scale can also be carried out by RNA interference (Travella et al. 2006), virus-induced gene silencing (Senthil-Kumar and Mysore 2011) or other related approaches which specifically target gene expression.

15.5.1 TILLING

TILLING (targeting-induced local lesions in genomes) is a reverse genetics method first described in *Arabidopsis* and *Drosophila* (Bentley et al. 2000; McCallum et al. 2000) and then extended to many other species, including most crops (Comai and Henikoff 2006). TILLING consists of DNA analysis of mutagenised individuals carrying a point mutation at a target gene. Point mutation discovery in TILLING is commonly based on an endonuclease enzyme that specifically recognises and cleaves at mismatch positions in double-stranded DNA molecules. The mismatches are produced in targeted amplicons obtained from individual DNA samples (or bulks) carrying both wild-type and mutated sequences. After PCR and enzymatic cleavage, electrophoretic approaches are utilised to separate and visualise the cut fragments, including acrylamide- or agarose-gel electrophoresis (Raghavan et al. 2007). Based on published protocols, enzymatic cleavage is obtained using CEL1, an endonuclease from celery or a non-purified celery leaf extract maintaining endonuclease activity (Till et al. 2012), or ENDO1, a similar endonuclease originally characterised in *A. thaliana* (Triques et al. 2007). Protocols can be adapted in order to exploit single- or double-strand DNA cuts (Uauy et al. 2009). A different, electrophoresis-free approach for identifying samples carrying mutations in TILLING is high-resolution melting curve analysis (HRM; Dong et al. 2009; Botticella et al. 2011; Shu et al. 2012). In HRM, an individual (or a DNA bulk) carrying a point mutation is identified based on the amplicon melting temperature shift induced by the presence of the mutation, relatively to a wild-type amplicon.

Direct sequencing of PCR products can also be utilised in TILLING provided that it is possible to discriminate sequencing errors from real mutations. Updating this approach, Rigola et al. (2009) and Tsai et al. (2011) took advantage of the NGS high-throughput capability to sequence multiple, different PCR target amplicons from multidimensional DNA pools in tomato, wheat and rice TILLING populations. Mutation-carrying individuals are identified in a single experiment. Additionally, accuracy and detection power of NGS-based TILLING compared favourably with other established methods (Tsai et al. 2011). Considering the NGS technologies capacity and costs, this appears to be a promising approach to TILLING in the near future.

Ultimately, sequencing costs are expected to decrease at a level that even whole-genome re-sequencing of an entire TILLING population will become economically practical (Wang et al. 2012) at least for research purposes. In this way, all mutations in any gene will be exposed and catalogued. Therefore, it is well possible that in the future, TILLING will be reduced to an in silico activity, requiring only the interrogation of database connecting molecular allelic variation of the target gene with the description of its mutant phenotypes and with the seed bank repository address. A shortcut in this direction has already been taken for a rice TILLING population, where mutations were identified in the exonic space by a combination of exome-capture and NGS technologies (Tsai et al. 2012).

Five TILLING resources have so far been fully described in barley, and two additional mutagenised populations obtained using MNU or γ-radiation were also produced in a TILLING perspective (Table 15.3); their mutation spectra is provided in Table 15.4. Populations were obtained using a range or even combinations of chemical mutagens and provided a mutation density from 1/300 to 1/2,500 (mutation/base pairs), which is in line with what was obtained for most diploid species (Wang et al. 2012). However, even at the highest mutation density, the probability of finding nonsense (i.e. truncation) or missense mutations which are informative for functional studies remains rather low. Indeed, a few thousand plants should be screened to find a potentially deleterious missense mutation with >90 % probability, and many more (tens of thousands) plants should be screened if a truncation is wanted (Parry et al. 2009; Wang et al. 2012). While TILLING populations of this size have been difficult to manage so far, it is possible that this will change with the use of NGS-based screening systems. Alternatively, and only speculatively at the moment, TILLING could also be attempted in polyploid barley since polyploid species were shown to accumulate tenfold or more mutations per genome (Wang et al. 2012). All barley TILLING resources described so far have been based on a traditional CEL1-LiCOR protocol. However, the TILLMore resource (www.distagenomics.unibo.it/TILLMore/) was recently switched to the HRM protocol for mutation screening. Internal comparative tests proved that the current HRM-based protocol reaches a sensitivity of at least 1 mutated copy out of 20 wild-type copies, equivalent to 1 heterozygous plant in a 10-plant bulk, which is the standard bulking level utilised in many TILLING populations.

Thanks to the possibility of recovering individuals carrying mutations (including knockouts) in a target gene, TILLING was also earlier proposed as an efficient aid to breeding (Slade and Knauf 2005). In this perspective, TILLING would enable the identification of mutations which are predicted to impact favourably on target traits. One important advantage is that TILLING-produced genotypes are not regulated by law as genetically modified. On the other hand, the mutant lines still require multiple backcross cycles in order to reduce the mutation load caused by off-target mutations. Additionally, mutagenesis mostly produces loss-of-function alleles; thereby, the range of phenotypes eventually produced is expected to be limited. This notwithstanding examples of TILLING-derived mutants of potential breeding interest are becoming available in different crops (Parry et al. 2009; Botticella et al. 2011; Bovina et al. 2011b; Slade et al. 2012; Wang et al. 2012).

Table 15.3 Summary of main parameters for TILLING populations developed in barley

Cultivar/ line	Mutagen	Dose	Size of M_2 population[a] (no.)	Genes (no.)	Screened sequence (Mb)	Detected mutations (no.)	Mutations per gene (no.)		Mutation density (kb per mutation)	Reference
							Average	Range		
Optic	EMS	20–30 mM/ 16 h	9,216	2	12.3	10	5.0	4–6	1,000	Caldwell et al. (2004)
Barke	EMS	20–60 mM/ 16 h	10,279 (7,389)	6	52.3	81	13.5	–	500[b]	Gottwald et al. (2009)
Morex[c]	NaN_3	10 mM/2 h	4,906 (3,148–*4,107*)	4 (*11*)	10.2 (*35.6*)	22 (*69*)	5.5 (*6.2*)	3–10	374[b] (*428*[b])	Talamè et al. (2008, 2009)
Lux	NaN_3	2.5 mM/2.5 h	9,575	2	12.3	5	2.5	2–3	2,500	Lababidi et al. (2009)
Sebastian	NaN_3 + MNU	1.5 mM/3 h + 0.5–0.7 mM/3 h	9,781 (3,935)	12	45.9	181	15.1	4–34	254	Szurman-Zubrzycka pers. comm.
DH 930-36	MNU	0.5–1.5 mM/ 3 h	1,372 (1,348)	2	4.5	9	4.5	3–6	504	Kurowska et al. (2012)
DH 930-36	Gamma rays	150–210 Gy	1,753 (1,644)	1	3.2	1	1.0	–	3,207	Kurowska et al. (2012)

[a]Actual number of analysed plants/families is given in brackets when different from population size
[b]100–200 bp of each amplicon was excluded from the calculation of the total sequence length used for estimation of mutation density
[c]In italics, updated results are provided after Talamè et al. (2009)

Table 15.4 Mutation types and spectrum in barley TILLING populations

Cultivar/ line	Mutagen	Mutations detected (no.)	Mutation spectrum				Substitution types			Reference
			Missense (%)	Silent (%)	Nonsense (%)	Non-coding (%)	Transitions G/C > A/T (%)	Transitions A/T > G/C (%)	Transversions (%)	
Optic	EMS	10	60	40	0	0	70	10	20	Caldwell et al. (2004)
Barke	EMS	81	35.8	39.5	3.7[a]	21	n.a.	n.a.	n.a	Gottwald et al. (2009)
DH 930-36	MNU	9	55.6	22.2	0	22.2	55.5	0	44.5	Kurowska et al. (2012)
Morex	NaN_3	69	56.6	23.2	1.4	18.8	97.1	0	2.9	Talamè et al. (2008, 2009)
Lux	NaN_3	5	60	0	0	40	80	0	20	Lababidi et al. (2009)
Sebastian	NaN_3/ MNU	181	43.6	35.4	1.7	19.3	89	4.4	6.6	Szurman-Zubrzycka pers. comm.
DH 930-36	Gamma	1	–	100	0	0	0	0	100	Kurowska et al. (2012)

n.a. data not available

[a]Nonsense and mutations in splice sites resulting in truncated protein

15.5.2 Insertional Mutagenesis

Insertional mutagenesis is increasingly becoming an effective tool for mining the fast-accumulating amount of sequence information in genomics studies. In this approach, a known DNA sequence is introduced into the genome under investigation and not only acts as a mutagenic factor but also conveniently tags the genetic variation occurred. Two strategies have been successfully applied in plant species: *Agrobacterium*-mediated T-DNA integration (Krysan et al. 1999) and transposon engineering and mobilisation (Ramachandran and Sundaresan 2001). The transposon systems so far utilised in heterologous systems are the *Activator*/*Dissociation* (*Ac*/*Ds*), the *Enhancer*/*Suppressor* (*En*/*Spm*) families and the *Mutator* (*Mu*) element of maize. Insertional mutagenesis can also be classified either as 'loss of function' or 'gain of function', accordingly if the insertional mechanism is merely designed to interrupt the gene coding sequence or if a specific alteration of the target gene expression, linked with a gene-reporter mechanism, is searched (Kuromori et al. 2009).

T-DNA insertions are generally stable, although often multicopy and characterised by inversion or truncation. Additionally, an easily applicable genetic transformation protocol must be available for the species, and this is not generally true for barley, except when the cv 'Golden Promise' is used (Hensel et al. 2008; Harwood 2012; see also Chap. 21). On the contrary, transposon-based insertional mutagenesis is more advantageous in species where genetic transformation is not easy since only a few initial transformation events are required in order to generate a larger number of insertional mutations, thanks to the mobilisation properties of transposons. Of course, a well-described and suitable transposon system must be available, either from the same species or a heterologous one. Both T-DNA and *Ac*/*Ds* type of transposons were shown to preferentially target genic regions for insertion (Zhao et al. 2006; Thole et al. 2012), which is a very important feature when dealing with species with large and repetitive genomes such as barley. Of relevance for cereal genetics, large resources of tagged lines (either by T-DNA or transposons) have been developed in *Brachypodium*, rice and maize (Greco et al. 2001; Kolesnik et al. 2004; Settles et al. 2007; Thole et al. 2012).

Based on the points described above and initial empirical evidence, the heterologous *Ac*/*Ds* two-element system appears today the most promising method for insertional mutagenesis in barley (McElroy et al. 1997; Koprek et al. 2000; Scholz et al. 2001; Cooper et al. 2004). The first relatively large set of barley *Ds*-insertion lines with mapped *Ds* elements was developed (Zhao et al. 2006). These materials can be used to generate a larger set of insertion mutants by *Ds* remobilisation. Genes in the proximity of the original *Ds* insertions will be targeted, thanks to the propensity of *Ds* elements to transpose to regions closely linked to the excision site (Singh et al. 2006). Conversely, when aiming to create a whole-genome insertion line collection, preferential short-range transposition appears as a limiting factor unless a very large number of starting lines are produced. *Ds* remobilisation has been obtained by two approaches, namely, by conventional crossing of the original

Ds-carrying line with an *AcTPase*-expressing parent and by genetic transformation of the *Ds*-carrying line with a construct carrying the *AcTPase* gene. The latter approach unexpectedly showed a higher remobilisation rate, perhaps due to the alteration of the chromatin methylation pattern occurring during the in vitro cultivation phase (Singh et al. 2012). Interestingly, Singh et al. (2006) reported a very high insertion rate (86 %) in genic regions as observed in maize and rice, while Zhao et al. (2006) observed some tendency for *Ds* elements to target low-copy, matrix-attachment regions.

Activation tagging and gene trapping are two other insertional mutagenesis-based approaches for plant functional genomics. In the first approach, a highly active promoter or enhancer is located on the T-DNA or the transposon, and following insertion of this element close to a gene, its expression is altered. Besides the possibility of generating knockouts, the deriving mutants are often dominant, providing the opportunity to produce gain-of-function phenotypes. Additionally, this approach enables to study genes belonging to partially functionally redundant gene families. In barley, an activation-tagging resource by random insertion of a modified *Ds* element containing two maize *polyubiquitin* promoters has been produced (Ayliffe et al. 2007; Ayliffe and Pryor 2011). For the second approach (gene trapping), a reporter gene is inserted and tracked based on its expression responding to regulatory regions close to the insertion site. Three different trapping systems (enhancer trap, promoter trap and gene trap) have been implemented in cereals. A promoterless or a minimal promoter associated to a reporter gene (*GUS*, *GFP*, *luciferase*, etc.) is usually utilised for transformation. The insertion events close to specifically regulated genes (e.g. stress conditions, developmental stages, etc.) can be identified by monitoring the expression of the reporter gene (Springer 2000). Lazarow and Lütticke (2009) reported an *Ac*/*Ds*-based gene trap system in barley exploiting a *Ds*-*GUS* fusion element. Stable and heritable expression of *GUS* linked with target genes was detected in a significant fraction of transformants and verified in different tissues.

15.5.3 Gene Targeting

Approaches to direct genetic changes to a particular gene comprise targeted mutagenesis and gene replacement. In the first case, the aim is to create localised, single-point mutations, whereas the existing target sequence is substituted with another one in the second case (Carroll 2011). While gene targeting is routinely applied in yeast and mice (Rothstein 1983; Capecchi 2005), and is rather advanced in moss *Physcomitrella* (Roberts et al. 2011), its application in crops has been difficult (Husaini et al. 2011). Two recently introduced approaches, the zinc-finger nuclease (ZFN) (Carroll 2011) and the transcription activator-like (TAL) effector proteins (Bogdanove and Voytas 2011), exploit enzymatic complexes acting on DNA. In both approaches, the enzymatic DNA-binding motifs were shown to be highly specific to particular target DNA sequences and flexible enough to be directed

(after modification of their amino acid sequence with genetic engineering) to alternative targets. Additional DNA-cleavage domains prompt the mutagenic action on target sequences by activating the endogenous DNA-break repairing systems. As these systems have already been shown to work in plants (Shukla et al. 2009; Mahfouz and Li 2011), a potential role in barley reverse genetics and genetic improvement can easily be predicted.

Conclusions
The developments in the field of genomics and NGS are potentially enabling us to investigate the genetic basis of a large number of barley mutants at an unprecedented speed. Additionally, functional genomics tools ranging from TILLING to insertional mutagenesis to targeted mutagenesis and/or gene substitution (the latter expected in the near future) are making gene function testing easier and, in some cases, can move reverse genetics to targeted genetic improvement. It should be finally reminded that in a situation where the molecular analyses are no longer a limiting step, advanced phenomics technologies which enable us to precisely and quickly phenotype many plants for many relevant traits are required in order to reduce and eventually close the phenotype-genotype gap (Houle et al. 2010). With all these tools at hand, barley mutants remain a valuable resource as precious and informative as before.

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References

Abe T, Ryuto H, Fukunishi M (2012a) Ion beam radiation mutagenesis. In: Shu QJ, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 99–106

Abe A, Kosugi S, Yoshida K, Natsume S, Takagi H, Kanzaki H, Matsumura H, Yoshida K, Mitsuoka C, Tamiru M, Innan H, Cano L, Kamoun S, Terauchi R (2012b) Genome sequencing reveals agronomically important loci in rice using MutMap. Nat Biotechnol 30:174–178

Ayliffe MA, Pryor AJ (2011) Activation tagging and insertional mutagenesis in barley. Methods Mol Biol 678:107–128

Ayliffe MA, Pallotta M, Langridge P, Pryor AJ (2007) A barley activation tagging system. Plant Mol Biol 64:329–347

Bamshad MJ, Ng SB, Bigham AW, Tabor HK, Emond MJ, Nickerson DA, Shendure J (2011) Exome sequencing as a tool for Mendelian disease gene discovery. Nat Rev Genet 12:745–755

Bentley A, MacLennan B, Calvo J, Dearolf CR (2000) Targeted recovery of mutations in *Drosophila*. Genetics 156:1169–1173

Blaby-Haas CE, de Crécy-Lagard V (2011) Mining high-throughput experimental data to link gene and function. Trends Biotechnol 29:174–182

Bogdanove AJ, Voytas DF (2011) TAL effectors: customizable proteins for DNA targeting. Science 333:1843–1846

Botticella E, Sestili F, Hernandez-Lopez A, Phillips A, Lafiandra D (2011) High resolution melting analysis for the detection of EMS induced mutations in wheat *SbeIIa* genes. BMC Plant Biol 11:156

Bovina R, Talamè V, Ferri M, Tuberosa R, Chmielewska B, Szarejko I, Sanguineti MC (2011a) Identification of root morphology mutants in barley. Plant Genet Resour 9:357–360

Bovina R, Talamè V, Salvi S, Sanguineti MC, Trost P, Sparla F, Tuberosa R (2011b) Starch metabolism mutants in barley: a TILLING approach. Plant Genet Resour 9:170–173

Bruce M, Hess A, Bai J, Mauleon R, Diaz MG, Sugiyama N, Bordeos A, Wang GL, Leung H, Leach JE (2009) Detection of genomic deletions in rice using oligonucleotide microarrays. BMC Genomics 10:129

Caldwell DG, McCallum N, Shaw P, Muehlbauer GJ, Marshall DF, Waugh R (2004) A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L.). Plant J 40:143–150

Capecchi MR (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 6:507–512

Carroll D (2011) Genome engineering with zinc-finger nucleases. Genetics 188:773–782

Chandler PM, Harding CA (2013) 'Overgrowth' mutants in barley and wheat: new alleles and phenotypes of the 'Green Revolution' *Della* gene. J Exp Bot 64:1603–1613. doi:10.1093/jxb/ert022

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney RK, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582

Comadran J, Kilian B, Russell J, Ramsay L, Stein N, Ganal M, Shaw P, Bayer M, Thomas W, Marshall D, Hedley P, Tondelli A, Pecchioni N, Francia E, Korzun V, Walther A, Waugh R (2012) A homologue of *Antirrhinum CENTRORADIALIS* is a component of the quantitative photoperiod and vernalization independent *EARLINESS PER SE 2* locus in cultivated barley. Nat Genet 44:1388–1392

Comai L, Henikoff S (2006) TILLING: practical single-nucleotide mutation discovery. Plant J 45:684–694

Cooper LD, Marquez-Cedillo L, Singh J, Sturbaum AK, Zhang S, Edwards V, Johnson K, Kleinhofs A, Rangel S, Carollo V, Bregitzer P, Lemaux PG, Hayes PM (2004) Mapping *Ds* insertions in barley using a sequence-based approach. Mol Genet Genomics 272:181–193

Costa JM, Corey A, Hayes PM, Jobet C, Kleinhofs A, Kopisch-Obusch A, Kramer SF, Kudrna D, Li M, Riera-Lizarazu O, Sato K, Szucs P, Toojinda T, Vales MI, Wolfe RI (2001) Molecular mapping of the Oregon Wolfe Barleys: a phenotypically polymorphic doubled-haploid population. Theor Appl Genet 103:415–424

Cuperus JT, Montgomery TA, Fahlgren N, Burke RT, Townsend T, Sullivan CM, Carringtonb JC (2010) Identification of *MIR390a* precursor processing-defective mutants in Arabidopsis by direct genome sequencing. Proc Natl Acad Sci U S A 107:466–471

Dolezel J, Greilhuber J, Lucretti S, Meister A, Lysák MA, Nardi L, Obermayer R (1998) Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann Bot 82:17–26

Dong C, Vincent K, Sharp S (2009) Simultaneous mutation detection of three homoeologous genes in wheat by high resolution melting analysis and mutation surveyor. BMC Plant Biol 9:143

Dormling I, Gustafsson Å, Jung HR, von Wettstein D (1966) Phytotron cultivation of Svalöf's Bonus barley and its mutant Svalöf's Mari. Hereditas 56:221–237

Druka A, Franckowiak J, Lundqvist U, Bonar N, Alexander J, Houston K, Radovic S, Shahinnia F, Vendramin V, Morgante M, Stein N, Waugh R (2011) Genetic dissection of barley morphology and development. Plant Physiol 155:617–627

Earley EJ, Jones CD (2011) Next-generation mapping of complex traits with phenotype-based selection and introgression. Genetics 189:1203–1209

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 6:e19379

Forster BP (2001) Mutation genetics of salt tolerance in barley: an assessment of Golden Promise and other semi-dwarf mutants. Euphytica 120:317–328

Forster BP, Franckowiak JD, Lundqvist U, Lyon J, Pitkethley I, Thomas WTB (2007) The barley phytomer. Ann Bot 100:725–733

Forster BP, Franckowiak JD, Lundqvist U, Thomas WTB, Leader D, Shaw P, Lyon J, Waugh R (2012) Mutant phenotyping and pre-breeding in barley. In: Shu QJ, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 327–346

Franckowiak JD, Lundqvist U (2009) Rules for nomenclature and gene symbolization in barley. Barley Genet Newsl 39:77–81

Gottwald S, Bauer P, Komatsuda T, Lundqvist U, Stein N (2009) TILLING in the two-rowed barley cultivar 'Barke' reveals preferred sites of functional diversity in the gene *HvHox1*. BMC Res Notes 2:258

Greco R, Ouwerkerk PBF, Sallaud C, Kohli A, Colombo L, Puigdomènech P, Guiderdoni E, Christou P, Hoge JHC, Pereira A (2001) Transposon insertional mutagenesis in rice. Plant Physiol 125:1175–1177

Gruszka D, Szarejko I, Maluszynski M (2012) Sodium azide as a mutagen. In: Shu QY, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 159–166

Harwood WA (2012) Advances and remaining challenges in the transformation of barley and wheat. J Exp Bot 63:1791–1798

Hensel G, Valkov V, Middlefell-Williams J, Kumlehn J (2008) Efficient generation of transgenic barley: the way forward to modulate plant-microbe interactions. J Plant Physiol 165:71–82

Houle D, Govindaraju DR, Omholt S (2010) Phenomics: the next challenge. Nat Rev Genet 11:855–866

Houston K, Druka A, Bonar N, Macaulay M, Lundqvist U, Franckowiak J, Morgante M, Stein N, Waugh R (2012) Analysis of the barley bract suppression gene *Trd1*. Theor Appl Genet 125:33–45

Husaini AM, Rashid Z, Mir RU, Aquil B (2011) Approaches for gene targeting and targeted gene expression in plants. GM Crops 2:150–162

International Barley Genome Sequencing Consortium (IBGSC) (2012) A physical, genetical and functional sequence assembly of the barley genome. Nature 491:711–716

Jackson SA, Iwata A, Lee SH, Schmutz J, Shoemaker R (2011) Sequencing crop genomes: approaches and applications. New Phytol 191:915–925

Kharkwal MC (2012) A brief history of plant mutagenesis. In: Shu QJ, Forster BP, Nakagawa H (eds) Plant Mutation Breeding and Biotechnology. CABI, Wallingford, pp 21–30

Kharkwal MC, Shu QY (2009) The role of induced mutations in world food security. In: Shu QY (ed) Induced plant mutations in the genomics era. Food and Agriculture Organization of the United Nations, Rome, pp 33–38

Kleinhofs A, Owais WM, Nilan RA (1978) Azide. Mutat Res 55:165–195

Kodym A, Afza R, Forster BP, Ukai Y, Nakagawa H, Mba C (2012) Methodology for physical and chemical mutagenic treatments. In: Shu QJ, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 169–180

Kolesnik T, Szeverenyi I, Bachmann D, Kumar CS, Jiang S, Ramamoorthy R, Cai M, Ma ZG, Sundaresan V, Ramachandran S (2004) Establishing an efficient *Ac/Ds* tagging system in rice: large-scale analysis of *Ds* flanking sequences. Plant J 37:301–314

Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Matsumoto T, Yano M (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci U S A 104:1424–1429

Koornneef M (2002) Classical mutagenesis in higher plants. In: Gilmartin PM, Bowler C (eds) Molecular plant biology. Oxford University Press, Oxford, pp 1–10

Koprek T, McElroy D, Louwerse J, Williams-Carrier R, Lemaux PG (2000) An efficient method for dispersing *Ds* elements in the barley genome as a tool for determining gene function. Plant J 24:253–263

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in Arabidopsis. Plant Cell 11:2283–2290

Kuromori T, Takahashi S, Kondou Y, Shinozaki K, Matsui M (2009) Phenome analysis in plant species using loss-of-function and gain-of-function mutants. Plant Cell Physiol 50:1215–1231

Kurowska M, Labocha-Pawłowska A, Gnizda D, Maluszynski M, Szarejko I (2012) Molecular analysis of point mutations in a barley genome exposed to MNU and gamma rays. Mutat Res 738–739:52–70

Lababidi S, Mejlhede N, Rasmussen SK, Backes G, Al-Said W, Baum M, Jahoor A (2009) Identification of barley mutants in the cultivar 'Lux' at the *Dhn* loci through TILLING. Plant Breed 128:332–336

Lazarow K, Lütticke S (2009) An *Ac*/*Ds*-mediated gene trap system for functional genomics in barley. BMC Genomics 10:55

Leitao JM (2012) Chemical mutagenesis. In: Shu QJ, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 135–158

Li G, Gelernter J, Kranzler HR, Zhao H (2012) M3: an improved SNP calling algorithm for Illumina BeadArray data. Bioinformatics 28:358–365

Lundqvist U (1992) Mutation research in barley. Dissertation, Department of Plant Breeding Research, The Swedish University of Agricultural Sciences, pp 11–38

Lundqvist U (2005) The Swedish collection of barley mutants held at the Nordic Genebank. Barley Genet Newsl 35:150–154

Lundqvist U (2009) Eighty years of Scandinavian barley mutation genetics and breeding. In: Shu QY (ed) Induced plant mutations in the genomics era. Food and Agriculture Organization of the United Nations, Rome, pp 39–43

Lundqvist U, Franckowiak J, Konishi T (1997) New and revised descriptions of barley genes. Barley Genet Newsl 26:22–44

Magori S, Tanaka A, Kawaguchi M (2010) Physically induced mutation: ion beam mutagenesis. In: Kahl G, Meksem K (eds) The handbook of plant mutation screening: mining of natural and induced alleles. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp 1–16

Mahfouz MM, Li L (2011) TALE nucleases and next generation GM crops. GM Crops 2:99–103

Maluszynski M, Szarejko I, Maluszynska J (2003) Mutation techniques. In: Thomas B, Murphy DJ, Murray BG (eds) Encyclopedia of applied plant sciences. Elsevier, San Diego, CA, pp 186–201

Mascher M, Richmond TA, Gerhardt DJ, Himmelbach A, Clissold L, Sampath D, Ayling S, Steuernagel B, Pfeifer M, D'Ascenzo M, Akhunov ED, Hedley PE, Gonzales AM, Morrell PL, Kilian B, Blattner FR, Scholz U, Mayer KF, Flavell AJ, Muehlbauer GJ, Waugh R, Jeddeloh JA, Stein N (2013) Barley whole exome capture: a tool for genomic research in the genus Hordeum and beyond. Plant J 76:494–505

Mascher M, Jost M, Kuon JE, Himmelbach A, Aßfalg A, Beier S, Scholz U, Graner A, Stein N (2014) Mapping-by-sequencing accelerates forward genetics in barley. Genome Biol 15:R78

Mayer KF, Martis M, Hedley PE, Simková H, Liu H, Morris JA, Steuernagel B, Taudien S, Roessner S, Gundlach H, Kubaláková M, Suchánková P, Murat F, Felder M, Nussbaumer T, Graner A, Salse J, Endo T, Sakai H, Tanaka T, Itoh T, Sato K, Platzer M, Matsumoto T, Scholz U, Dolezel J, Waugh R, Stein N (2011) Unlocking the barley genome by chromosomal and comparative genomics. Plant Cell 23:1249–1263

Mba C, Afza R, Shu QY (2012) Mutagenic radiations: X-rays, ionizing particles and ultraviolet. In: Shu QJ, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 83–90

McCallum CM, Comai L, Greene EA, Heinkoff S (2000) Targeting Induced Lesions INGenomes (TILLING) for plant functional genomics. Plant Physiol 123:439–442

McElroy D, Louwerse JD, McEIroy SM, Lemaux PG (1997) Development of a simple transient assay for *Ac*/*Ds* activity in cells of intact barley tissue. Plant J 11:157–165

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci U S A 88:9828–9832

Muller HJ (1927) Artificial transmutation of the gene. Science 66:84–87

Pankin A, Campoli C, Dong X, Kilian B, Sharma R, Himmelbach A, Saini R, Davis SJ, Stein N, Schneeberger K, von Korff M (2014) Mapping-by-sequencing identifies HvPHYTOCHROME C as a candidate gene for the early maturity 5 locus modulating the circadian clock and photoperiodic flowering in barley. Genetics (in press)

Parry MAJ, Madgwick PJ, Bayon C, Tearall K, Hernandez-Lopez A, Baudo M, Rakszegi M, Hamada W, Al-Yassin A, Ouabbou H, Labhilili M, Phillips AL (2009) Mutation discovery for crop improvement. J Exp Bot 60:2817–2825

Petr J, Lipavsky J, Hradecká D (2002) Production process in old and modern spring barley varieties. Die Bodenkultur 53:19–27

Pozzi C, di Pietro D, Halas G, Roig C, Salamini F (2003) Integration of a barley (Hordeum vulgare) molecular linkage map with the position of genetic loci hosting 29 developmental mutants. Heredity 90:390–396

Raghavan C, Naredo M, Wang H, Atienza G, Liu B, Qiu F, McNally K, Leung H (2007) Rapid method for detecting SNPs on agarose gels and its application in candidate gene mapping. Mol Breed 19:87–101

Ramachandran S, Sundaresan V (2001) Transposons as tools for functional genomics. Plant Physiol Biochem 39:243–252

Ramsay L, Comadran J, Druka A, Marshall DF, Thomas WT, Macaulay M, MacKenzie K, Simpson C, Fuller J, Bonar N, Hayes PM, Lundqvist U, Franckowiak JD, Close TJ, Muehlbauer GJ, Waugh R (2011) *INTERMEDIUM-C*, a modifier of lateral spikelet fertility in barley, is an ortholog of the maize domestication gene *TEOSINTE BRANCHED 1*. Nat Genet 43:169–172

Rigola D, van Oeveren J, Janssen A, Bonné A, Schneiders H, van der Poel HJA, van Orsouw NJ, Hogers RCJ, de Both MTJ, van Eijk MJT (2009) High-throughput detection of induced mutations and natural variation using KeyPoint™ technology. PLoS One 4:e4761

Roberts AW, Dimos CS, Budziszek MJ Jr, Goss CA, Lai V (2011) Knocking out the wall: protocols for gene targeting in *Physcomitrella patens*. Methods Mol Biol 715:273–290

Romero Loli M, Gomez Pando L (2001) Barley breeding in Peru. In: Vivar HE, McNab A (eds) Breeding barley in the new millenium, Proceedings of an international symposium. CYMMIT, Mexico, DF

Rostoks N, Ramsay L, MacKenzie K, Cardle L, Bhat PR, Roose ML, Svensson JT, Stein N, Varshney RK, Marshall DF, Graner A, Close TJ, Waugh R (2006) Recent history of artificial outcrossing facilitates whole-genome association mapping in elite inbred crop varieties. Proc Natl Acad Sci U S A 103:18656–18661

Rothstein RJ (1983) One-step gene disruption in yeast. Method Enzymol 101:202–211

Schneeberger K, Weigel D (2011) Fast-forward genetics enabled by new sequencing technologies. Trends Plant Sci 16:282–288

Schneeberger K, Ossowski S, Lanz C, Juul T, Petersen AH, Nielsen KL, Jørgensen JE, Weigel D, Andersen SU (2009) SHOREmap: simultaneous mapping and mutation identification by deep sequencing. Nat Methods 6:550–551

Scholz S, Lörz H, Lütticke S (2001) Transposition of maize transposable element *Ac* in barley (*Hordeum vulgare* L.). Mol Gen Genet 264:653–661

Senthil-Kumar M, Mysore KS (2011) New dimensions for VIGS in plant functional genomics. Trends Plant Sci 16:656–665

Settles AM, Holding DR, Tan BC, Latshaw SP, Liu J, Suzuki M, Li L, O'Brien BA, Fajardo DS, Wroclawska E, Tseung CW, Lai J, Hunter CT 3rd, Avigne WT, Baier J, Messing J, Hannah LC, Koch KE, Becraft PW, Larkins BA, McCarty DR (2007) Sequence-indexed mutations in maize using the *UniformMu* transposon-tagging population. BMC Genomics 8:116

Shu QY, Shirasawa K, Hoffmann M, Hurlebaus J, Nishio T (2012) Mutation techniques and methods for mutation detection and screening in plants. In: Shu QY, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 241–256

Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X, Choi VM, Rock JM, Wu YY, Katibah GE, Zhifang G, McCaskill D, Simpson MA, Blakeslee B, Greenwalt SA, Butler HJ, Hinkley SJ, Zhang L, Rebar EJ, Gregory PD, Urnov FD (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459:437–441

Šimková H, Svensson JT, Condamine P, Hribová E, Suchánková P, Bhat PR, Bartos J, Safár J, Close TJ, Dolezel J (2008) Coupling amplified DNA from flow-sorted chromosomes to high-density SNP mapping in barley. BMC Genomics 9:294

Singh J, Zhang S, Chen C, Cooper L, Bregitzer P, Sturbaum A, Hayes PM, Lemaux PG (2006) High-frequency *Ds* remobilization over multiple generations in barley facilitates gene tagging in large genome cereals. Plant Mol Biol 62:937–950

Singh S, Tan HQ, Singh J (2012) Mutagenesis of barley malting quality QTLs with *Ds* transposons. Funct Integr Genomics 12:131–141

Slade AJ, Knauf VC (2005) TILLING moves beyond functional genomics into crop improvement. Transgenic Res 14:109–115

Slade AJ, McGuire C, Loeffler D, Mullenberg J, Skinner W, Fazio G, Holm A, Brandt KM, Steine MN, Goodstal JF, Knauf VC (2012) Development of high amylose wheat through TILLING. BMC Plant Biol 12:69

Springer PS (2000) Gene traps: tools for plant development and genomics. Plant Cell 12:1007–1020

Sreenivasulu N, Graner A, Wobus U (2008) Barley genomics: an overview. Int J Plant Genomics 2008:486258

Stadler LJ (1928) Mutations in barley induced by X-rays and radium. Science 68:186–187

Stadler LJ (1930) Some genetic effect of X-rays in plants. J Hered 21:3–19

Szarejko I, Maluszynski M (1980) Translocations and inversions in barley induced by fast neutrons and N-nitroso-N-methylurea (MNUA). Barley Genet Newsl 10:67–69

Talamè V, Bovina R, Sanguineti MC, Tuberosa R, Lundqvist U, Salvi S (2008) TILLMore, a resource for the discovery of chemically induced mutants in barley. Plant Biotechnol J 6:477–485

Talamè V, Bovina R, Salvi S, Sanguineti MC, Piffanelli P, Lundquist U, Tuberosa R (2009) TILLING with TILLMore. In: Shu QY (ed) Induced plant mutations in the genomics era. Food and Agriculture Organization of the United Nations, Rome, pp 240–242

Tanaka A, Shikazano N, Hase Y (2010) Studies on biological effects of ion beams on lethality, molecular nature of mutation, mutation rate, and spectrum of mutation phenotype for mutation breeding in higher plants. J Radiat Res 51:223–233

Teo YY (2012) Genotype calling for the Illumina platform. Method Mol Biol 850:525–538

Thole V, Peraldi A, Worland B, Nicholson P, Doonan JH, Vain P (2012) T-DNA mutagenesis in *Brachypodium distachyon*. J Exp Bot 63:567–576

Till BJ, Zerr T, Comai L, Henikoff S (2012) A protocol for TILLING and Eco-TILLING. In: Shu QY, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI, Wallingford, pp 269–286

Travella S, Klimm TE, Keller B (2006) RNA interference based gene silencing as an efficient tool for functional genomics in hexaploid bread wheat. Plant Physiol 142:7–20

Trick M, Adamski NM, Mugford SG, Jiang CC, Febrer M, Uauy C (2012) Combining SNP discovery from next-generation sequencing data with bulked segregant analysis (BSA) to fine-map genes in polyploid wheat. BMC Plant Biol 12:14

Triques K, Sturbois B, Gallais S, Dalmais M, Chauvin S, Clepet C, Aubourg S, Rameau C, Caboche M, Bendahmane A (2007) Characterization of *Arabidopsis thaliana* mismatch specific endonucleases: application to mutation discovery by TILLING in pea. Plant J 51:1116–1125

Tsai H, Howell T, Nitcher R, Missirian V, Watson B, Ngo KJ, Lieberman M, Fass J, Uauy C, Tran RK, Khan AA, Filkov V, Tai TH, Dubcovsky J, Comai L (2011) Discovery of rare mutations in populations: TILLING by sequencing. Plant Physiol 156:1257–1268

Tsai H, Nitcher R, Howell TR, Akhunov E, Tai TH, Dubcovsky J, Comai L (2012) High-throughput sequencing makes TILLING more fun. Plant and Animal Genome Conference XX, San Diego, CA, W217

Uauy C, Paraiso F, Colasuonno P, Tran RK, Tsai H, Berardi S, Comai L, Dubcovsky J (2009) A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. BMC Plant Biol 9:115

Uchida N, Sakamoto T, Kurata T, Tasaka M (2011) Identification of EMS-induced causal mutations in a non-reference *Arabidopsis thaliana* accession by whole genome sequencing. Plant Cell Physiol 52:716–722

van Harten AM (1998) Induction of mutation. In: van Harten AM (ed) Mutation breeding: theory and practical applications. Cambridge University Press, Cambridge, pp 111–160

van Oeveren J, de Ruiter M, Jesse T, van der Poel H, Tang J, Yalcin F, Janssen A, Volpin H, Stormo KE, Bogden R, van Eijk MJ, Prins M (2011) Sequence-based physical mapping of complex genomes by whole genome profiling. Genome Res 21:618–625

von Korpf M, Leon J, Pillen K (2010) Detection of epistatic interactions between exotic alleles introgressed from wild barley (*H. vulgare* ssp. *spontaneum*). Theor Appl Genet 121:1455–1464

Wang L, Wang A, Huang X, Zhao Q, Dong G, Qian Q, Sang T, Han B (2011) Mapping 49 quantitative trait loci at high resolution through sequencing-based genotyping of rice recombinant inbred lines. Theor Appl Genet 122:327–340

Wang TL, Uauy C, Robson F, Till B (2012) TILLING *in extremis*. Plant Biotechnol J 10:761–772

Waugh R, Leader DJ, McCallum N, Caldwell D (2006) Harvesting the potential of induced biological diversity. Trends Plant Sci 11:71–79

White PJ, Bengough AG, Bingham IJ, George TS, Karley AJ, Valentine TA (2009) Induced mutations affecting root architecture and mineral acquisition in barley. In: Shu QY (ed) Induced plant mutations in the genomics era. Food and Agriculture Organization of the United Nations, Rome, pp 338–340

Wienholds E, van Eeden F, Kosters M, Mudde J, Plasterk RHA, Cuppen E (2003) Efficient target-selected mutagenesis in zebrafish. Genome Res 13:2700–2707

Zakhrabekova S, Gough SP, Braumann I, Müller AH, Lundqvist J, Ahmanna K, Dockter C, Matyszczak I, Kurowska M, Druka A, Waugh R, Graner A, Stein N, Steuernagel B, Lundqvist U, Hansson M (2012) Induced mutations in circadian clock regulator *Mat*-a facilitated short-season adaptation and range extension in cultivated barley. Proc Natl Acad Sci U S A 109:4326–4331

Zhao T, Palotta M, Langridge P, Prasad M, Graner A, Schulze-Lefert P, Koprek T (2006) Mapped *Ds*/T-DNA launch pads for functional genomics in barley. Plant J 47:811–826

Chapter 16
Modulation of Meiotic Recombination

Luke Ramsay, Isabelle Colas, and Robbie Waugh

16.1 Introduction

Meiotic recombination is one of the principal forces creating the genetic diversity that drives evolution and is the fundamental instrument underlying most crop breeding programmes. A greater understanding of the control of recombination in crop plants would enable manipulation of this process to improve the speed and accuracy of plant breeding (Riley et al. 1981; Able et al. 2009; Martinez-Perez and Moore 2008). This would be particularly useful for many of the temperate grass species (e.g. wheat, barley, oats and forage grasses such as Lolium and Festuca) where the highly skewed distribution of meiotic crossover (CO) events means that a large proportion of genes rarely, if ever, recombine. These species are all members of the grass subfamily Pooideae, with large genomes and very close syntenic relationships (Moore et al. 1995), and all exhibit a non-random pattern of recombination relative to the gene distribution in their genomes whereby chiasmata appear to be preferentially targeted to the ends of the chromosomes. This means that large areas of the chromosome around the centromeric region rarely recombine, even though they represent substantial proportions of the physical maps of the chromosomes (Künzel et al. 2000; Künzel and Waugh 2002). Thus, the genes in such areas are inherited together as large linkage blocks, preventing the generation of novel allele combinations and useful variation that could be exploited in breeding programmes (Rostoks et al. 2006). Even small changes in the crossover frequency and distribution, particularly those that promote recombination in centromeric regions, could therefore have a significant effect on the efficiency of breeding in these crops by breaking up some of the extensive linkage blocks (Fig. 16.1). Achieving this objective is not unrealistic as partial loss of control of chiasmata distribution can occur in certain inbred lines of normally outbred species

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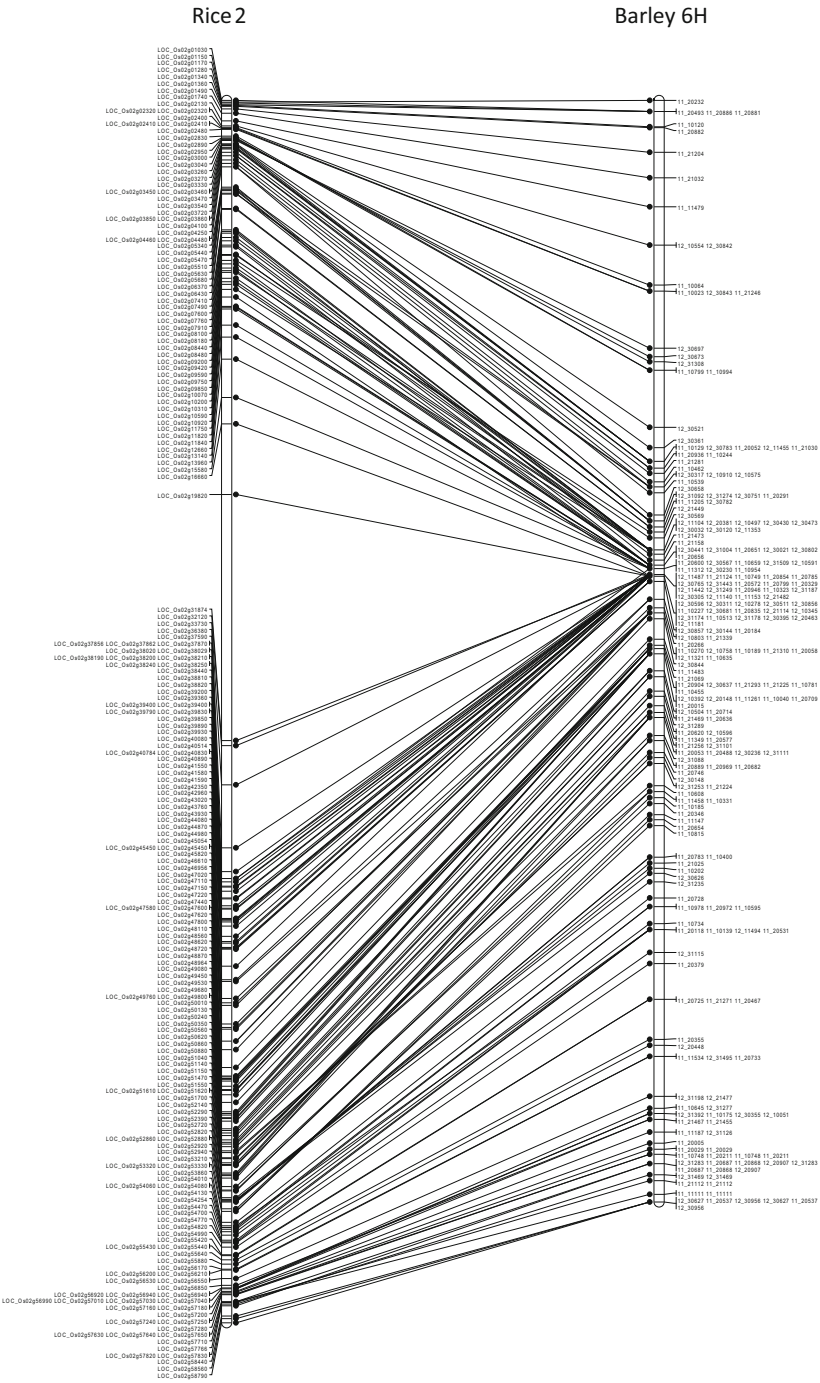

Fig. 16.1 Comparison of the physical map of rice chromosome 2 and the genetic map of barley chromosome 6H. Alignment of rice chromosome 2 and barley chromosome 6H through the position of genetic SNPs on the barley genetic map demonstrating colinearity and the variation in the recombination rate

such as rye and Lolium (Jones 1967; Karp and Jones 1983). Barley offers critical natural advantages for studies on the control of recombination in crop plants, being an inbreeding diploid that benefits from a wide range of genetic and genomic resources.

16.2 Recombination Frequency and Distribution in Barley

Barley has a large genome of 5.3 Mb that is contained within seven pairs of chromosomes with a well-recognised karyotype (Linde-Laursen 1997). The genetic maps of this species show extensive colinearity with other crop species in the Triticeae and Pooideae (Dubcovsky et al. 1996; Jones et al. 2002). This colinearity extends further to sequenced species such as rice and Brachypodium, though the syntenic relationship is more complicated (Close et al. 2009). Recent studies have indicated that the genetic map length of these sequenced model species is similar with the map of rice totalling 1,500 cM and that of Brachypodium measuring 1,570 cM (Harushima et al. 1998; Huo et al. 2011). Recent genetic mapping of barley has indicated that the map length is considerably shorter, averaging 1,100 cM, indicating that the gene complement in rice undergoes potentially 35 % more recombination over time than in barley. This map length would reflect an average of 22 crossovers (CO) per cell during meiosis given that each crossover only involves two of the four chromatids of the bivalent. The map length of barley has been a matter of some dispute (Nilsson et al. 1993) as historically crossover number has been inferred from chiasmata counts of metaphase I spreads that typically display seven ring bivalents that have been taken to indicate the presence of 14 crossovers (Gale and Rees 1970). The difference between the crossover frequency inferred from genetic mapping and that inferred from chiasmata counts has become less with improvements in the quality of marker technology that have reduced the map length found in genetic mapping studies. However, the difference remains, even with the advent of high-throughput SNP assays (Close et al. 2009) which have generated maps with a high density of very robust markers. The difference is potentially due to the difficulties in discriminating physically close chiasmata in metaphase I spreads, and this is now being investigated with antibodies raised to the MutL homologue 3 protein (MLH3). MLH3 is a DNA mismatch repair protein involved in homologous recombination and meiosis (Jackson et al. 2006) that forms a heterocomplex with MLH1 which can be visualised as foci that mark the position of crossovers after homologous recombination at pachytene (Flores-Rozas and Kolodner 1998; Wang and Kung 2002) that give potentially a more accurate cytological estimation of the CO numbers that are subject to interference (Lhuissier et al. 2007).

The presence of ring bivalents at metaphase I indicates a strongly distal distribution of chiasmata and such a pattern of recombination has been supported by the use of genic SNPs in high-density genetic maps that give information on the patterns of recombination relative to gene content (Close et al. 2009). The patterns

observed explain the clustering of marker loci found at the 'centromeric' regions of genetic maps with a range of marker technologies and confirm the inferences made from early comparative mapping with RFLP (Künzel et al. 2000). The comparison of physical and genetic mapping has confirmed that the distribution of recombination is highly skewed to the distal ends of the chromosomes, considerably more skewed than that of the distribution of genes (Mayer et al. 2011). Barley shows a less pronounced correlation of recombination and gene content than observed in rice and Brachypodium (International Rice Genome Sequencing Project 2005; The International Brachypodium Initiative 2010). This disjunction between recombination and gene content in barley that means that up to half of the genes are within genomic regions that rarely, if ever, recombine. Comparative genetic mapping evidence indicates that this pattern is conserved across most of the Pooideae (Brachypodium being the exception).

There have been a number of studies on recombination frequency in barley. Gale and Rees (1970) found some differences in chiasmata counts in a range of material and through a diallel analysis with a limited number of morphological markers, found evidence that some of the genetic variation shown for the trait of chiasmata number could be assigned to chromosome 2H. Säll et al. (1990) found significant differences in the recombination frequency of a number of varieties through the degree of sterility in F_2 plants resulting from a cross with an inversion stock (a genetic line that carries an inversion that prevents recombination in that region).

The advent of molecular markers for genetic mapping enabled many more comparisons to be made between maps albeit using smaller population sizes. Comparison of doubled haploid populations made using anther culture and the *Hordeum bulbosum* technique derived from the same cross showed little reciprocal difference between maps derived from male and female meiosis, respectively (Cistué et al. 2011; Devaux et al. 1995). The use of high-throughput SNP assays (Close et al. 2009) has allowed more detailed comparisons between maps given the improved coverage and improved quality of the underlying data. A comparison of three maps (Morex × Barke , OWB and Steptoe × Morex) that underpinned construction of a broadly used consensus map indicated compete conservation of linear order but some differences in map length of particular chromosomes (Fig. 16.2) (Close et al. 2009).

Some differences could be seen within specific regions of the maps with, for example, the mapping interval around one of the genes involved in the control of inflorescence architecture, the intermedium-c gene (*int-c*) on 4HS, being significantly smaller in polymorphic two-rowed by six-rowed crosses (Morex × Barke and OWB) than the interval in the six-rowed by six-rowed crosses (Steptoe × Morex) which was conversely largely monomorphic.

Such inverse relationship between polymorphism and recombination could partly explain some of the differences observed in wider crosses. An indication of reduced recombination in the centromeric regions was found in a comparison of a wide cross involving a *H. v. spontaneum* parent with more standard mapping populations (Ramsay et al. 2000). However, more fundamental differences in genetic control could also be involved. A comparison of RFLP maps derived

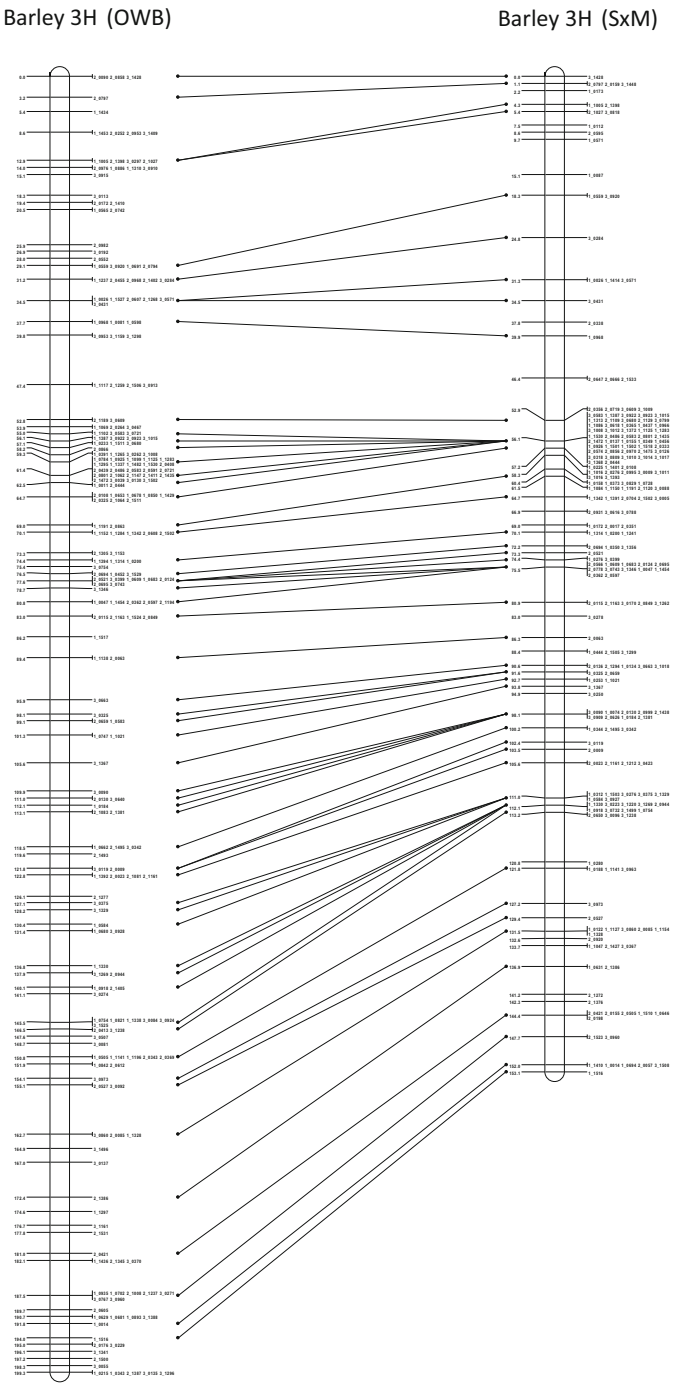

Fig. 16.2 Comparison of recombination patterns in the 3H genetic maps derived from two different mapping populations. Alignment of common SNP loci between the 3H genetic maps derived from the Oregon Wolfe Barley and Steptoe × Morex DH mapping populations. The two

from a *Hordeum bulbosum* cross indicated even stronger distal distribution of recombination than in standard *H. vulgare* maps (Salvo-Garrido et al. 2001) corresponding to similar differences between out- and inbreeding species in the Triticeae (Dvorak et al. 1998).

The indication that the distribution of recombination is under genetic control is supported by the findings of Gale and Rees (1970) and later studies that have used whole genome approaches to identify genes affecting recombination using a QTL approach within segregating mapping populations derived from a biparental cross (Esch et al. 2007). This uses a score of the recombination events evident in each individual of the population as a quantitative trait that can be used in a standard QTL analysis albeit with limited resolution. This relies upon the segregation of the controlling QTL in previous generations to generate the phenotype observed, and thus, DH population derived from F_1 material are not appropriate material for such investigations (Bovill et al. 2009). QTL have been found for recombination in a number of the RIL populations studied by Bovill et al. (2009) that highlighted in particular a region on 6HL, and the authors indicated a number of candidate genes through synteny with rice chromosome 2 that could underlie the QTL observed, though the region covered roughly 30 cM on the consensus map.

Patterns of recombination have long been known to be affected by environmental factors (Plough 1917) which could potentially complicate the comparisons made between mapping populations generated at different times (Devaux et al. 1995; Cistué et al. 2011). In barley and related species such as wheat, a number of older studies using chiasmata counts to estimate recombination frequency showed significant effects due to abiotic stress. In particular, the use of mutant and cytological stocks that allowed the effects to be more clearly manifested showed that both temperature and nutrient stress influence chiasmata frequency (Fedak 1973; Fu and Sears 1973). The effect of abiotic stress on meiosis has also been studied at the transcript level (Oshino et al. 2007), and interactions between environmental and developmental effects have been shown by partial recovery of temperature-induced sterility through the exogenous application of the plant hormone auxin (Sakata et al. 2010).

16.3 Meiosis

An understanding of the control of recombination forms part of the process of meiosis which is central to eukaryotic sexual reproduction. During meiosis, DNA replication is followed by two rounds of division (meiosis I and meiosis II) which produces four haploid cells from one original diploid cell—fertilisation restores the

Fig. 16.2 (continued) maps show excellent colinearity but varying genetic distances between some loci and different total map lengths

diploid state. Each round of division has four steps: prophase, metaphase, anaphase and telophase, with prophase I itself being subdivided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis, which are distinguished from each other by chromatin organisation and specific mechanisms such as the telomere mobility (Alberts et al. 2008).

At leptotene, chromatin starts to condense, and the chromosomes are visible as long threads with the sister chromatids tightly associated together. During zygotene, the chromosomes become thicker, and the telomeres cluster in a region of the nuclear envelope to form a structure called the 'bouquet' which is believed to promote synapsis (Bass 2003). Synapsis is the association of the homologues which occurs in a zipper-like manner, resulting in the two homologous chromosomes being side by side, and the chromosome pair at this stage is called a bivalent. The synaptonemal complex (SC) that is fully formed at pachytene disappears at diplotene when the homologues separate to be held only via chiasmata, as a result of recombination events (Zickler and Kleckner 1998).

Recombination during prophase I of meiosis results in genetic crossover (CO) formation which establishes physical links between the homologous chromosomes. The controlled formation of these COs is dependent on coordination of the recombination process with extensive remodelling of the chromosomes occurring during meiotic prophase I.

Subsequently, when recombination events are completed and the synaptonemal complex disassembles, the chromosomes are held together only by chiasmata (the cytogenetic manifestation of the crossovers at metaphase I with the bivalents aligned at the equatorial plate). Each of the homologous chromosomes then separates, and segregation occurs to each pole at anaphase I to enter the second round of cell division resulting in sister chromatid separation and the formation of haploid cells. The precise mechanisms and timing of key events such as chromosome recognition, synapsis and importantly recombination are still under debate in the field, and current research has concentrated on model eukaryotic species such as yeast, mouse and *C. elegans*.

Considerable progress has been made in the translation of this fundamental knowledge to plants through the use of Arabidopsis and rice. *Arabidopsis thaliana* in particular has been of great help in understanding plant meiosis and recombination using a combined approach in genetics and cytology (Armstrong et al. 2003, 2009; Jones and Franklin 2007; Jones et al. 2003). The large number of Arabidopsis T-DNA insertion lines and the use of reverse genetics (Caryl et al. 2003) have contributed to the identification of key players during meiosis and recombination (De Muyt et al. 2009; Osman et al. 2011). This research led to the development of enabling research tools, such as antibodies raised against meiosis-specific proteins, that have proved invaluable for functional analysis studies (Armstrong et al. 2002; Higgins et al. 2005; Sanchez-Moran et al. 2008).

Although Arabidopsis has dominated, crop species with large genomes have provided new insights in meiosis research and plant science in general, as demonstrated in wheat, barley and maize (Boden et al. 2009; Colas et al. 2008; Franklin et al. 1999; Jasencakova et al. 2001; Mikhailova et al. 2006; Phillips et al. 2010;

Prieto et al. 2004; Wang et al. 2009). Cytogenetic studies using these plants have contributed to genomics projects—helping to delineate markers, aligning genetic and physical maps and revealing genome organisation (Bass and Bircher 2011; Figueroa and Bass 2010). However, with improved chromosome preparation, cytology protocols and microscopy technologies, new possibilities now exist for cytogenetic research. Taking advantage of large genome species (wheat, maize, barley), methods to image 3D meiocytes using epifluorescent or confocal microscopes have been developed (Aragon-Alcaide et al. 1998; Franklin et al. 1999; Mikhailova et al. 2006; Prieto et al. 2007). Recent improvements to structured illumination microscopy allowing an increased spatial resolution of wide-field fluorescence microscopy have enabled the application of this technique to chromosome organisation such as in the clear resolution of lateral elements of the synaptonemal complex in maize (Carlton 2008; Gustafsson et al. 2008; Wang et al. 2009). Such structured illumination microscopes are now commercially available and represent a highly enabling tool for plant cytology (Dobbie et al. 2011).

It is estimated that the length of meiosis in barley is around 40 h from prophase to telophase II with little variation between cultivar or between male and female meiosis. Moreover, anthers of the same florets are synchronised in wheat and barley with approximately the same size for each meiotic stage, making it possible to sample specific meiotic stages with reasonable accuracy (Bennett et al. 1973; Bennett and Finch 1971; Ekberg and Eriksson 1965). Checks using a combination of acetocarmine chromosome spreads and anther size to estimate the stage of meiosis allow accurate chromosome counts and configurations to be made at metaphase I. Barley generally exhibits seven ring bivalents at metaphase I (Fig. 16.3a), resulting in equal reduction of the homologues at anaphase I (Fig. 16.3b) and fully fertile pollen (Fig. 16.3c). Importantly, a series of semi-sterile 'desynaptic' mutants have been isolated and superficially characterised at the cytological level in barley (Hernandez-Soriano and Ramage 1973). These show abnormal metaphases as exemplified by *des7* (Fig. 16.3d–f) and exhibit varying number of rod bivalents and univalents at metaphase I (Fig. 16.3d) resulting in aberrant segregation and chromosome loss (Fig. 16.3e) leading to infertile pollen (Fig. 16.3f) and semi-sterility.

The antibodies raised in Arabidopsis have proved an invaluable tool to study chromosome pairing and synapsis in rye (Mikhailova et al. 2006), and the use of specific DNA probes, antibodies and confocal microscopy has been very powerful in meiosis research, for wheat, maize and barley (Colas et al. 2008; Franklin et al. 1999; Jasencakova et al. 2001; Phillips et al. 2010; Prieto et al. 2007). However, to study recombination and crossovers, electron microscopy (EM) has been the only method with sufficient resolution to identify each single strand. The disadvantage of EM is that fluorochromes cannot be used and therefore protein colocalisation cannot be easily assessed. However, a new platform, the OMX 3D-SIM, now offers resolution that approaches that of EM, with the advantage that the same sample prepared for confocal images can be used. For example, in Fig. 16.4, barley meiocytes at different stages of prophase from the barley cultivar

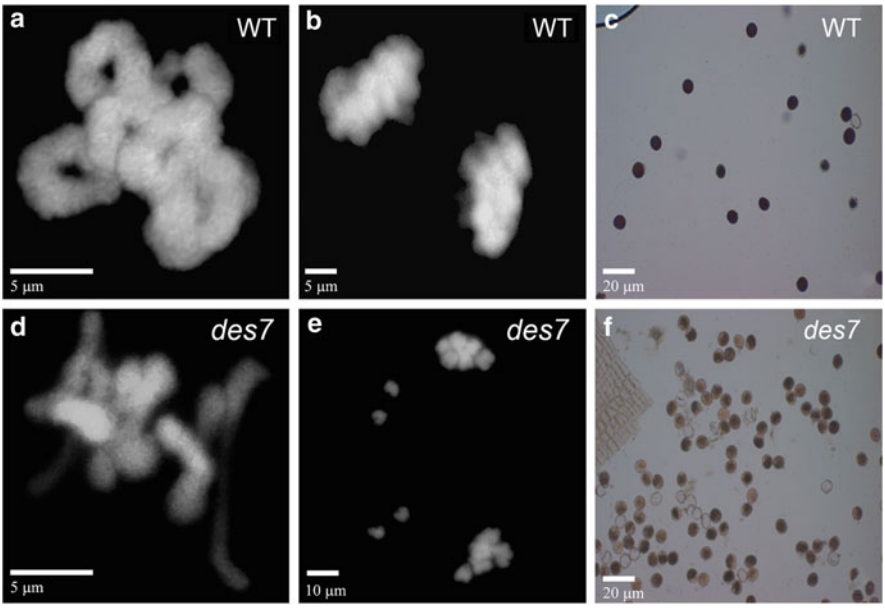

Fig. 16.3 Chromosome spread and pollen staining of wild-type barley and the desynaptic mutant *des7*. Wild-type metaphase I (**a**) showing seven ring bivalents as the result of chiasmata at the end of each chromosome arm with the chromosomes attached to the metaphase plate. However, *des7* (**d**) exhibits open or rod bivalents as well as univalents because of a reduction in chiasmata formation. At anaphase I, the chromosomes cluster and correctly segregate in the wild type (**b**) resulting in two balanced cell containing half the genetic complement, whilst in *des7*, chromosomes can be lost (**e**) leading to unbalanced gametes. After acetocarmine staining, viable pollen is dark in the wild-type spread (**c**), but the pollen in *des7* is either dark, lightly stained or empty due to the results of meiotic resolution of unbalanced gametes (**f**), leading to semi-sterility

Bowman were fixed, labelled with fluorescent antibodies raised against the proteins asynaptic 1 (ASY1) and zipper-like 1 (ZYP1) and images taken with the OMX (DeltaVision).

At the initiation of synapsis in leptotene (Fig. 16.4a, d, g), the chromosomes are fully coated with the protein ASY1 (Fig. 16.a, g) whilst the protein ZYP1 is localised at one side of the nucleus (Fig. 16.4d, g). This is where homologous chromosomes are brought together at the beginning of zygotene (Fig. 16.4b, e, h) before the chromosome strands progressively to pair up in a zipper-like manner (Fig. 16.4e, h) until synapsis is complete in pachytene (Fig. 16.4c, f, i, l). Whilst with a confocal microscope, both ASY1 and ZYP1 signals would colocalise, 3D-SIM imaging enables clearer discrimination of each channel and the localisation of the regions currently paired up (white arrow in Fig. 16.4g, h, i). The distance between the two homologous chromosomes held by ZYP1 when the synaptonemal complex is formed is about 109 nm which is in the range of the 3D-SIM resolution. Therefore, this technique enables us to see the two ASY1-labelled chromosome strands perfectly aligned along their lengths (Fig. 16.4c, i)

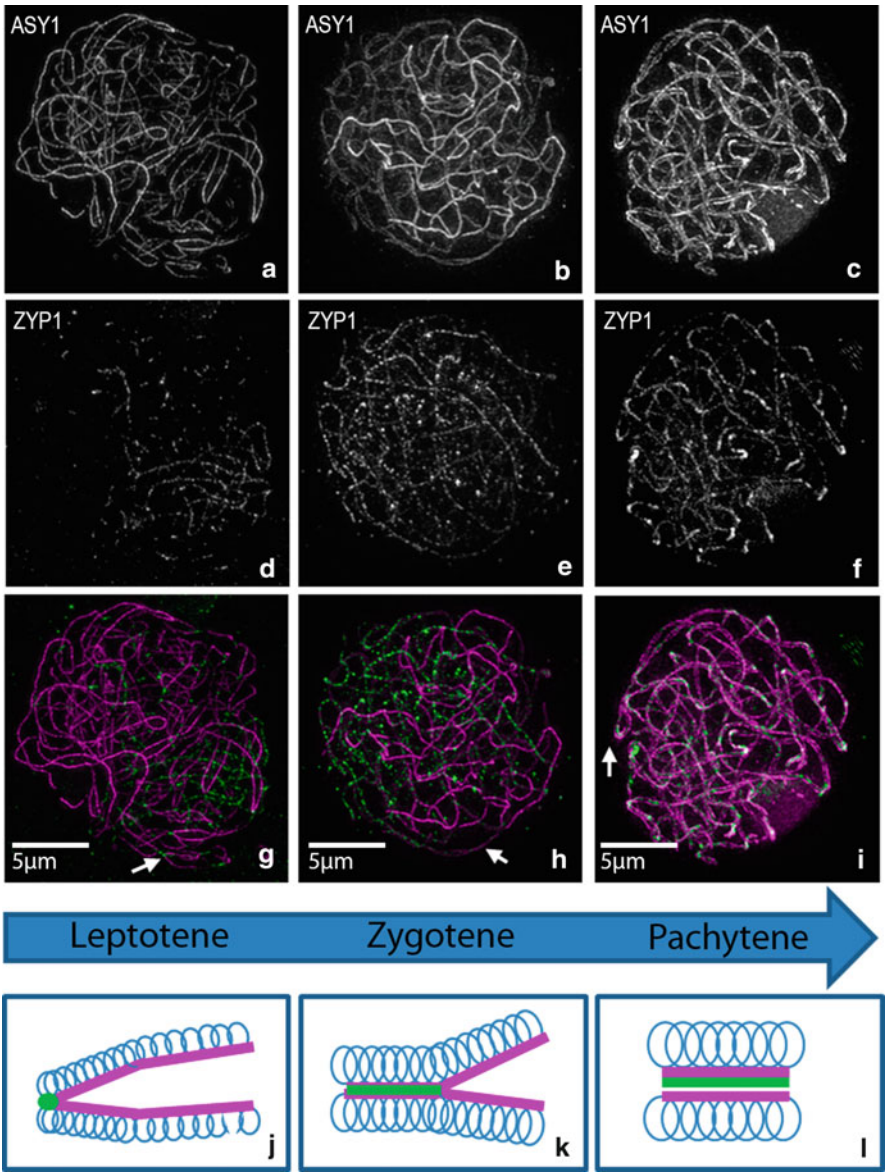

Fig. 16.4 Synapsis progression in barley. Analysis of synapsis progression using structure illumination microscopy at a 100 nm resolution with dual immunolocalisation with ASY1 (*magenta* channel **a**, **b**, **c**) and ZYP1 (*green* channel **d**, **e**, **f**) enabling the visualisation of the synaptonemal complex (SC) (merged channels **g**, **h**, **i**; cartoon **j**, **k**, **l**) (scale bar 5 µm). At leptotene (**a**, **d**, **g**, **j**), chromosomes' axes are fully prepared for synapsis and fully coated with ASY1 (**a**, **g**), homologous chromosomes are brought together via ZYP1 (**d**, **g**) and few sites are already perfectly aligned (*arrow* in **g**). By mid-zygotene (**b**, **e**, **h**, **k**), chromosomes are synapsing in a zipper-like manner with a bright ASY1 signal (**b**) indicative of unsynapsed chromosomes, whereas the linearisation of ZYP1 indicates the synapsed chromosomal regions (**e**). The SC starts to be recognisable with the two chromosomes linked via ZYP1 in most of the nucleus (**h**, *arrow*). At pachytene (**c**, **f**, **i**, **l**) homologous chromosomes are synapsed along their entire length (**c**, **i**, **l**) via ZYP1 (**f**, **i**, **l**), and the SC is clearly visible (**i**, *arrow*)

and tightly held together by the protein ZYP1 (Fig. 16.4f, i). OMX images also reveal that both signals are not completely linear, potentially representing individual protein complexes or different chromatin states with varying degrees of condensation.

16.4 Manipulation of Recombination

Given the historical, genetic and genomic resources now available in barley together with the modern cytogenetic approaches and the rapidly developing understanding of the processes underlying meiosis and recombination, there exists the potential for being able to manipulate recombination in barley and other large genome cereals.

16.4.1 Reverse Genetic Approaches

There is now the possibility of using candidate genes to manipulate recombination in barley using a reverse genetic approach and information on the process of meiosis that has been generated from yeast, mammals and *C. elegans* and transferred to Arabidopsis. This is feasible in barley as robust and efficient protocols are now available to transform barley using *Agrobacterium tumefaciens* and enable functional analysis of targeted genes (Bartlett et al. 2008; Harwood et al. 2009). The mismatch repair (MMR) system undertaken by MutH and MutL homologues (MSH and MHL, respectively) is essential for DNA repair. During meiosis, MSH proteins (except MSH1) are involved in DNA repair and/or recombination whilst suppressing homoeologous recombination. In barley, RNAi has been successfully used to downregulate MSH7 (Lloyd et al. 2007). MSH7 is specific to plants and forms a heterodimer with MSH2 involved in controlling homoeologous recombination. In wheat, MSH7 maps on the short arm of the group 3 chromosomes where the homoeologous suppressor Ph2 lies. Using the wheat sequences *TaMSH7*, RNAi knockdowns of barley have been successfully produced. The loss of MSH7 function led to loss of fertility with a reduction of seed set and the overall size of the grain.

The synapsis initiation complex called ZMM (Zip1, Zip2, Zip3, Zip4, Mer3 (merlin gene allele 3) and Msh4/Msh5) genes known to be required for recombination and synapsis have been identified in barley and have been targeted for the use of RNAi in barley in ongoing investigations. These are initial studies that have been designed to test the commonality of meiotic control in large genome cereals with that in the model systems. The phenotypes observed in most of these initial studies, though informative for the genetic dissection of the trait, are not necessarily those desired for the manipulation of recombination in a desired fashion for applications in breeding. Indeed, although considerable progress has been made in the understanding of the control of recombination and interference in model systems (Osman et al. 2011), the number of potential candidates that could be prioritised for

manipulation is limited. The recent identification of *Fanconi anaemia complementation group M* (*FANCM*) in Arabidopsis and the elucidation of its role in the control of recombination (Crismani et al. 2012; Knoll et al. 2012) provide an attractive candidate for such an approach, though its effect in large genome cereals is as yet unknown. In particular it is as yet an open question whether Arabidopsis can provide good candidate genes to alter the skewed distribution of recombination in barley rather than just affecting the frequency within the existing distribution given the peculiar skewed distribution phenotype does not exist in the model system.

16.4.2 Forward Genetic Approaches

Barley is fortunate in having a considerable collection of characterised morphological mutants (Franckowiak et al. 2005) that include a number of desynaptic mutants that show aberrant meiotic behaviour (Hernandez-Soriano and Ramage 1973). Desynaptic mutants have been defined as 'those mutants in which pairing between homologous chromosomes occurs at early stages of prophase but the chromosomes fail to remain paired in the later stages' compared to asynaptic mutants that do not pair at all (Li et al. 1945). Such mutants can be found in many plants species including other cereals such as maize, rye and rice and are valuable tools for functional analysis of the mechanism of meiosis (Maguire et al. 1991; Reddi and Srao 2000).

In maize, the desynaptic mutants *dy* and *dsy1* have a novel telomere-misplacement phenotype (Bass et al. 2003) with the three *dsy1* alleles exhibiting a partial telomere bouquet formation at zygotene and effects on the fidelity and progression of homologous synapsis whilst the *dy* mutant seems to have a normal bouquet but an abnormal dispersion of the telomeres at pachytene in addition to a reduced recombination rate. The rye meiotic mutant collection has been investigated genetically and cytologically with the mutants being classified into six groups involving different meiotic mechanisms: (1) nonallelic asynaptic mutations (s*y1*, *sy9*), (2) weak asynaptic mutation (*sy3*), (3) partially non-homologous synapsis at prophase I (*sy2*, *sy6*, *sy7*, *sy8*, *sy10* and *sy1*), (*4*) synaptonemal complex (SC) ultrastructural alteration (*mei6*), (5) irregular chromatin condensation along chromosome, sticking and fragmentation of chromosomes in metaphase I (*mei8* and *mei8-10*) and (6) chromosome hypercondensation, defects of the division spindle formation and random arrest of cells at different meiotic stages (*mei5* and *mei10*) (Mikhailova et al. 2010; Sosnikhina et al. 2005, 2007). In more recent studies using the rye mutant *sy10*, chromosome pairing has been described using dual immunolocalisation of the synaptic proteins ASY1 and ZYP1 during meiosis (as shown above for barley) that enabled the authors to conclude that the asynaptic phenotype was not the direct result of the failure to form the synaptonemal complex (Mikhailova et al. 2006). More importantly, this study revealed that synapsis progression in rye was somewhat different from Arabidopsis, suggesting that although mechanisms

are conserved at meiosis, and knowledge can be transferred, some features remain different from one organism to another.

In barley, a collection of meiotic mutants has been described based on their phenotype for male sterility and chromosome behaviour at metaphase I. The *sc* gene, standing for 'short' chromosome, exhibits super condensed chromosomes at metaphase I, whilst the 'long' chromosome, renamed later as *desynaptic 1* (*des1*), shows elongated rod bivalents (Burnham 1946; Moh and Nilan 1954). In 1960, Enns and Larter described a mutant (*des2*) where the chromosomes pair during prophase but failed to remain linked at metaphase I because of the lack of chiasmata (Enns and Larter 1960). Later desynaptic lines were identified in the cultivar Betzes and classified according to their 'degree of desynapsis' based on their cytological behaviour (Franckowiak et al. 2005; Hernandez-Soriano and Ramage 1973; Hernandez-Soriano 1973; Ramage and Hernandez-Soriano 1971, 1972). As part of a larger backcrossing programme, the 14 nonallelic desynaptic (*des*) mutants have also been backcrossed into a common background (cltv. Bowman), creating a series of near-isogenic lines (Druka et al. 2010). These Bowman isolines provide a unique resource for studying meiosis and the control of recombination in barley and form the basis of ongoing work that takes advantage of the advances in molecular cytology to determine more detailed differences between the mutants and with wild type. These barley mutants are thus being assessed for synapsis (using antibodies against ASY1 and ZYP1), recombination (using antibodies against the recombination proteins RAD51 and MLH3) and chromatin organisation (histone marks) using the knowledge and tools developed in Arabidopsis (Osman et al. 2011). These have highlighted differences between mutant phenotypes including effects on recombination that range from mild to severe. These studies are informing genetic mapping work that is underway in order to identify the genes involved. The severity of the mutant phenotypes with varying degrees of semi-sterility means that the mutations themselves are unlikely to have a direct application in breeding programmes, but they do provide a unique means of dissecting out the control of recombination and the progression of meiosis in this large genome cereal.

16.4.3 Other Approaches

The manipulation of recombination by non-genetic means would have considerable advantage in a breeding context if the timely application of a chemical or environmental stress could induce the desired effect. This would avoid the use of unadapted germ plasm that would be inevitable if a genetic approach was used (irrespective of whether a transgene or mutant allele was introduced) that would in turn necessitate a pre-breeding programme. The use of chemicals to induce double strand breaks or inhibit repair is under investigation in particular in the context of wheat genetics where recombination is additionally controlled by the action of the *pairing homologous 1* (*Ph1*) locus (Vorontsova et al. 2004). However although disruption of the meiotic phenotype has been achieved, the impact on the recombination patterns in

subsequent generations is as yet unknown, necessitating the recovery of viable seeds (Greer et al. 2012; Knight et al. 2010).

The use of temperature to manipulate recombination is also an active research area that holds promise given the known effects that have been found in past studies (Fu and Sears 1973). Interestingly more interstitial chiasmata at metaphase I have been observed after raising the temperature prior to meiosis (Higgins et al. 2012) that potentially could provide a means of modulating recombination within a breeding programme. The effect of the observed chiasmata on the recombination patterns in subsequent generations is currently being assessed through the use of a genome-wide SNP panel (Close et al. 2009). It is becoming clear that a critical time for the control of recombination is in the early stages of prophase, perhaps in the pre-meiosis interphase, a considerable time before the cytological manifestation of crossovers (Bayliss and Riley 1972). The recent demonstration of the involvement of the Arabidopsis homologue of the cell-cycle control protein retinoblastoma (pRb) in the control of recombination in Arabidopsis (Chen et al. 2011) supports the importance of the very early stages of meiosis for recombination as well as pairing. Given the lack of a full understanding of the genetic control of this early stage, a more empirical approach using environmental factors to manipulate recombination has many attractions.

Conclusion

All breeding work relies fundamentally on the process of recombination. Thus a deeper understanding of the control of this process during meiosis offers the opportunity to manipulate recombination to profoundly improve the speed and accuracy of plant breeding in order to address future needs of food security within increased environmental constraints. This is particularly important in large genome species such as barley where there is a disjunction between the patterns of recombination and gene content. As an inbreeding diploid, barley offers many advantages for the investigation of the control of recombination given the advances in cytological techniques and the genomic and genetic resources now available in the species. Some advances have already been made using reverse and forward genetic approaches and the use of abiotic stress. However, it yet remains to be seen if such methodology will give breeders the ability to fundamentally alter the recombination distribution in barley and access the considerable proportion of the genome that is at present largely inherited as linkage blocks. However, even the progress already made offers the possibility of manipulation of recombination frequency within the current distribution bounds which may already serve to relieve one of the fundamental constraints on breeding programme progress.

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References

Able JA, Crismai W, Boden SA (2009) Understanding meiosis and the implications for crop improvement. Funct Plant Biol 36:575–588

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008) Molecular biology of the cell, 5th edn. Garland Science/Taylor and Francis Group, New York, NY

Aragon-Alcaide L, Beven A, Moore G, Shaw P (1998) The use of vibratome sections of cereals spikelets to study anther development and meiosis. Plant J 1:503–508

Armstrong SJ, Caryl AP, Jones GH, Franklin FCH (2002) Asy1, a protein required for meiotic chromosome synapsis, localizes to axis-associated chromatin in Arabidopsis and Brassica. J Cell Sci 115:3645–3655

Armstrong SJ, Franklin FCH, Jones GH (2003) A meiotic time-course for Arabidopsis thaliana. Sex Plant Reprod 16:141–149

Armstrong SJ, Sanchez-Moran E, Franklin FCH (2009) Cytological analysis of Arabidopsis thaliana meiotic chromosomes. Methods Mol Biol 558:131–145

Bartlett JG, Alves SC, Smedley M, Snape JW, Harwood WA (2008) High-throughput Agrobacterium-mediated barley transformation. Plant Methods 4:22

Bass HW (2003) The conservation and divergence of telomeric structures, effects, and functions. Cell Mol Life Sci 60:2281–2282

Bass HW, Bircher JA (eds) (2011) Plant cytogenetics: genome structure and chromosome function. Springer, New York, NY

Bass HW, Bordoli SJ, Foss EM (2003) The desynaptic (dy) and desynaptic1 (dsy1) mutations in maize (*Zea mays* L) cause distinct telomere-misplacement phenotypes during meiotic prophase. J Exp Bot 54:39–46

Bayliss MW, Riley R (1972) Evidence of premeiotic control of chromosome pairing in Triticum aestivum. Genet Res 20:201–212

Bennett MD, Finch RA (1971) Duration of meiosis in barley. Genet Res 17:209–214

Bennett MD, Finch RA, Smith JB, Rao MK (1973) Time and duration of female meiosis in wheat, rye and barley. Proc R Soc Lond B 183:301–319

Boden SA, Langridge P, Spangenberg G, Able JA (2009) TaASY1 promotes homologous chromosome interactions and is affected by deletion of Ph1. Plant J 57:487–497

Bovill WD, Deveshwar P, Kapoor S, Able JA (2009) Whole genome approaches to identify early meiotic gene candidates in cereals. Funct Integr Genomics 9:219–229

Burnham CR (1946) A gene for "long" chromosome in barley. Genetics 31:212–213

Carlton PM (2008) Three-dimensional structured illumination microscopy and its application to chromosome structure. Chromosome Res 16:351–365

Caryl AP, Jones GH, Franklin FCH (2003) Dissecting plant meiosis using Arabidopsis thaliana mutants. J Exp Bot 54:25–38

Chen Z, Higgins JD, Hui JTL, Li J, Franklin FCH, Berger F (2011) Retinoblastoma protein is essential for early meiotic events in Arabidopsis. EMBO J 30:744–755

Cistué L, Cuesta-Marcos A, Chao S, Echávarri B, Chutimanitsaku Y, Corey A, Filichkina T, Garcia-Mariño N, Romagos I, Hayes PM (2011) Comparative mapping of the Oregon Wolfe Barley using doubled haploid lines derived from female and male gametes. Theor Appl Genet 122:1399–1410

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roos ML, Moscou MJ, Varshney R, Chao S, Szücs P, Sato K, Hayes

PM, Matthews DE, Marshall DF, Muehlbauer GJ, Graner A, DeYoung J, Madishetty K, Fenton RD, Condamine P, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582

Colas I, Shaw P, Prieto-Aranda PP, Wanous MK, Spielmeyer W, Mago R, Moore G (2008) Effective chromosome pairing requires chromatin remodelling at the onset of meiosis. Proc Natl Acad Sci U S A 105:6075–6080

Crismani W, Girard C, Froger N, Pradillo M, Santos JL, Chelysheva L, Copenhaver GP, Horlow C, Mercier R (2012) FANCM limits meiotic crossovers. Science 336:1588–1590

De Muyt A, Pereira L, Vezon D, Chelysheva L, Gendrot G, Chambon A, Laine-Choinard S, Pelletier G, Mercier R, Nogue F, Grelon M (2009) A high throughput genetic screen identifies new early meiotic recombination functions in Arabidopsis thaliana. PLoS Genet 5:9

Devaux P, Kilian A, Kleinhofs A (1995) Comparative mapping of the barley genome with male and female recombination-derived, doubled haploid populations. Mol Gen Genet 249:600–608

Dobbie IM, King E, Parton RM, Carlton PM, Sedat JW, Swedlow JR, Davis I (2011) OMX: a new platform for multimodal, multichannel wide-field imaging. Cold Spring Harb Protoc 2011:899–909

Druka A, Franckowiak J, Lundqvist U, Bonar N, Alexander J, Guzy-Wrobelska J, Ramsay L, Druka I, Grant I, Macaulay M, Vendramin V, Shahinnia F, Radovic S, Houston K, Harrap D, Cardle L, Marshall D, Morgante M, Stein N, Waugh R (2010) Exploiting induced variation to dissect quantitative traits in barley. Biochem Soc Trans 38:683–688

Dubcovsky J, Luo MC, Zhong GY, Brandsteitter R, Desai A, Kilian A, Kleinhofs A, Dvorak J (1996) Genetic map of diploid wheat, *Triticum monococcum* L and its comparison with maps of *Hordeum vulgare* L. Genetics 143:983–999

Dvorak J, Luo MC, Yang ZL (1998) Restriction fragment length polymorphism and divergence in the genomic regions of high and low recombination in self-fertilizing and cross-fertilizing *Aegilops* species. Genetics 148:423–434

Ekberg I, Eriksson G (1965) Demonstration of meiosis and pollen mitosis by photomicrographs and the distribution of meiotic stages in barley spikes. Hereditas 53:127–136

Enns H, Larter EN (1960) Note on the inheritance of *ds*, a gene governing meiotic chromosome behavior in barley. Can J Plant Sci 40:570–571

Esch E, Szymaniak JM, Yates H, Pawlowsk WP, Buckler ES (2007) Using crossover breakpoints in recombinant inbred lines to identify quantitative trait loci controlling the global recombination frequency. Genetics 177:1851–1858

Fedak G (1973) Increased chiasma frequency in desynaptic barley in response to phosphate treatments. Can J Genet Cytol 15:647–649

Figueroa DM, Bass HW (2010) A historical and modern perspective on plant cytogenetics. Brief Funct Genomics 9:95–102

Flores-Rozas H, Kolodner RD (1998) The Saccharomyces cerevisiae MLH3 gene functions in MSH3-dependent suppression of frameshift mutations. Proc Natl Acad Sci U S A 95:12404–12409

Franckowiak J, Kleinhofs A, Lundqvist U (2005) Descriptions of barley genetic stocks for 2005. Barley Genet Newsl 35:155–210

Franklin AE, McElver J, Sunjevaric I, Rothstein R, Bowen B, Cande WZ (1999) Three-dimensional microscopy of the Rad51 recombination protein during meiotic prophase. Plant Cell 11:809–824

Fu TK, Sears ER (1973) The relationship between chiasmata and crossing over in Triticum aestivum. Genetics 75:231–246

Gale MD, Rees H (1970) Genes controlling chiasma frequency in *Hordeum*. Heredity 25:393–410

Greer E, Martin AC, Pendle A, Colas I, Jones AM, Moore G, Shaw P (2012) The Ph1 locus suppresses Cdk2-type activity during premeiosis and meiosis in wheat. Plant Cell 24:152–162

Gustafsson MG, Shao L, Carlton PM, Wang CJ, Golubovskaya IN, Cande WZ, Agard DA, Sedat JW (2008) Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. Biophys J 94:4957–4970

Harushima Y, Yano M, Shomura A, Sato M, Shimano T, Kuboki Y, Yamamoto T, Lin SY, Antonio BA, Parco A, Kajiya H, Huang N, Yamamoto K, Nagamura Y, Kurata N, Khush GS, Sasaki T (1998) A high-density rice genetic linkage map with 2275 markers using a single F_2 population. Genetics 148:479–494

Harwood WA, Bartlett JG, Alves SC, Perry M, Smedley MA, Leyland N, Snape JW (2009) Barley transformation using Agrobacterium-mediated techniques. Methods Mol Biol 478:137–147

Hernandez-Soriano JM (1973) Desynaptic mutants in Betzes barley. MS Thesis, University of Arizona, 1 Jan 1973

Hernandez-Soriano JM, Ramage RT (1973) Desynaptic genes. Barley Genet Newsl 4:91

Higgins JD, Sanchez-Moran E, Armstrong SJ, Jones GH, Franklin FCH (2005) The Arabidopsis synaptonemal complex protein ZYP1 is required for chromosome synapsis and normal fidelity of crossing over. Genes Dev 19:2488–2500

Higgins JD, Perry RM, Barakate A, Ramsay L, Waugh R, Halpin C, Armstrong SJ, Franklin FC (2012) Spatiotemporal asymmetry of the meiotic program underlies the predominantly distal distribution of meiotic crossovers in barley. Plant Cell 24:4096–4109

Huo N, Garvin DF, You FM, McMahon S, Luo MC, Gu YQ, Lazo GR, Vogel JP (2011) Comparison of a high-density genetic linkage map to genome features in the model grass Brachypodium distachyon. Theor Appl Genet 123:455–464

International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. Nature 436:793–800

Jackson N, Sanchez-Moran E, Buckling E, Armstrong SJ, Jones GH, Franklin FCH (2006) Reduced meiotic crossovers and delayed prophase I progression in AtMLH3-deficient *Arabidopsis*. EMBO J 25:1315–1323

Jasencakova Z, Meister A, Schubert I (2001) Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley. Chromosoma 11:643–651

Jones GH (1967) Control of chiasma distribution in rye. Chromosoma 22:69–90

Jones GH, Franklin FC (2007) Meiosis in Arabidopsis thaliana: recombination, chromosome organization and meiotic progression. In: Egel R, Lankenau D-H (eds) Recombination and meiosis. Spring, Berlin, pp 279–306

Jones ES, Mahoney NL, Hayward MD, Armstead IP, Jones JG, Humphreys MO, King IP, Kishida T, Yamada T, Balfourier F, Charmet G, Forster JW (2002) An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other Poaceae genomes. Genome 45:282–295

Jones GH, Armstrong SJ, Caryl AP, Franklin FC (2003) Meiotic chromosome synapsis and recombination in Arabidopsis thaliana; an integration of cytological and molecular approaches. Chromosome Res 11:205–215

Karp A, Jones RN (1983) Cytogenetics of Lolium perenne 2: chiasma distribution in inbred lines. Theor Appl Genet 64:137–145

Knight E, Greer E, Draeger T, Thole V, Reader S, Shaw P, Moore G (2010) Inducing chromosome pairing through premature condensation: analysis of wheat interspecific hybrids. Funct Integr Genomics 10:603–608

Knoll A, Higgins JD, Seeliger K, Reha SJ, Dangel NJ, Bauknecht M, Schröpfer S, Franklin FCH, Puchta H (2012) The fanconi anemia ortholog FANCM ensures ordered homologous recombination in both somatic and meiotic cells in *Arabidopsis thaliana*. Plant Cell 24:1448–1464

Künzel G, Waugh R (2002) Integration of microsatellite markers into the translocation-based physical RFLP map of barley chromosome 3H. Theor Appl Genet 105:660–665

Künzel G, Korzun L, Meister A (2000) Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics 154:397–412

Lhuissier FGP, Offenberg HH, Wittich PE, Vischer NOE, Heyting C (2007) The mismatch repair protein MLH1 marks a subset of strongly interfering crossovers in tomato. Plant Cell 19:862–876

Li HW, Pao WK, Li CH (1945) Desynapsis in the common wheat. Am J Bot 32:92–101

Linde-Laursen I (1997) Recommendations for the designation of the barley chromosomes and their arms. Barley Genet Newsl 26:1–3
Lloyd AH, Milligan AS, Langridge P, Able JA (2007) TaMSH7: a cereal mismatch repair gene that affects fertility in transgenic barley (Hordeum vulgare L). BMC Plant Biol 7:67
Maguire MP, Paredes AM, Riess RW (1991) The desynaptic mutant of maize has a combined defect of synaptonemal complex and chiasma maintenance. Genome 34:879–887
Martinez-Perez E, Moore G (2008) To check or not to check? The application of meiotic studies to plant breeding. Curr Opin Plant Biol 11:222–227
Mayer KFX, Martis M, Hedley PE, Šimková H, Liu H, Morris JA, Steuernagel B, Taudien S, Roessner S, Gundlach H, Kubaláková M, Suchánková P, Murat F, Felder M, Nussbaumer T, Graner A, Salse J, Endo T, Sakai H, Tanaka T, Itoh T, Sato K, Platzer M, Matsumoto T, Scholz U, Doležel J, Waugh R, Stein N (2011) Unlocking the barley genome by chromosomal and comparative genomics. Plant Cell 23:1249–1263
Mikhailova EI, Phillips D, Sosnikhina SP, Lovtsyus AV, Jones RN, Jenkins G (2006) Molecular assembly of meiotic proteins Asy1 and Zyp1 and pairing promiscuity in rye (Secale cereale L) and its synaptic mutant sy10. Genetics 174:1247–1258
Mikhailova EI, Lovtsius AV, Sosnikhina SP (2010) Some features of meiosis key events in rye and its synaptic mutants. Genetika 46:1371–1375
Moh CC, Nilan CC (1954) "Short" chromosome – a mutant in barley induced by atomic bomb irradiation. Cytologia (Tokyo) 19:48–53
Moore G, Devos KM, Wang Z, Gale MD (1995) Cereal genome evolution – grasses, line up and form a circle. Curr Biol 5:737–739
Nilsson N-O, Säll T, Bengtsson BO (1993) Chiasma and recombination data in plants: are they compatible? Trends Genet 9:344–348
Oshino T, Abiko M, Saito R, Ichiishi E, Endo M, Kawagishi-Kobayashi M, Higashitani A (2007) Premature progression of anther early developmental programs accompanied by comprehensive alterations in transcription during high-temperature injury in barley plants. Mol Genet Genomics 278:31–42
Osman K, Higgins JD, Sanchez-Moran E, Armstrong SJ, Frankli FC (2011) Pathways to meiotic recombination in Arabidopsis thaliana. New Phytol 190:523–544
Phillips D, Nibau C, Ramsay L, Waugh R, Jenkins G (2010) Development of a molecular cytogenetic recombination assay. Cytogenet Genome Res 129:154–161
Plough HH (1917) The effect of temperature on linkage in the second chromosome of Drosophila. Proc Natl Acad Sci U S A 3:553–555
Prieto P, Shaw P, Moore G (2004) Homologue recognition during meiosis is associated with a change in chromatin conformation. Nat Cell Biol 6:906–908
Prieto P, Moore G, Shaw P (2007) Fluorescence in situ hybridization on vibratome sections of plant tissues. Nat Protoc 2:1831–1838
Ramage RT, Hernandez-Soriano J (1971) Desynaptic genes in Betzes barley. Barley Genet Newsl 1:38
Ramage RT, Hernandez-Soriano J (1972) Desynaptic genes in barley. Barley Genet Newsl 2:65–68
Ramsay L, Macaulay M, Ivanissevich SD, MacLean K, Cardle L, Fuller JD, Edwards KJ, Tuvesson S, Morgante M, Massari A, Maestri E, Marmiroli N, Sjakste T, Ganal M, Powell W, Waugh R (2000) A simple sequence repeat-based linkage map of barley. Genetics 156:1997–2005
Reddi TVV, Srao DRM (2000) Cytology of induced desynaptic mutants in rice. Cytologia 65:35–41
Riley R, Law CN, Chapman V (1981) The control of recombination. Phil Trans R Soc Lond B 292:529–534
Rostoks N, Ramsay L, MacKenzi K, Cardle L, Bhat PL, Roose ML, Svensson JT, Stein N, Varshney RK, Marshall D, Graner A, Close TJ, Waugh R (2006) A recent history of artificial

outcrossing facilitates whole genome association mapping in elite inbred crop varieties. Proc Natl Acad Sci U S A 103:18656–18661

Sakata T, Oshino T, Miura S, Tomabechi M, Tsunaga Y, Higashitani N, Miyazawa Y, Takahashi H, Watanabe M, Higashitani A (2010) Auxins reverse plant male sterility caused by high temperatures. Proc Natl Acad Sci U S A 107:8569–8574

Säll T, Flink J, Bengtsson BO (1990) Genetic control of recombination in barley I Variation in recombination frequency measured with inversion heterozygotes. Hereditas 112:157–170

Salvo-Garrido H, Laurie DA, Jaffé B, Snape JW (2001) An RFLP map of diploid Hordeum bulbosum L and comparison with maps of barley (H. vulgare L) and wheat (Triticum aestivum L). Theor Appl Genet 103:869–880

Sanchez-Moran E, Osman K, Higgins JD, Pradillo M, Cunado N, Jones GH, Franklin FC (2008) ASY1 coordinates early events in the plant meiotic recombination pathway. Cytogenet Genome Res 120:302–312

Sosnikhina SP, Mikhailova EI, Tikholiz OA, Priiatkina SN, Smirnov VG, Voilokov AV, Fedotova I, Kolomiets OL, Bogdanov I (2005) Genetic collection of meiotic mutants of rye Secale cereale L. Genetika 41:1310–1321

Sosnikhina SP, Mikhailova EI, Tikholiz OA, Tsvetkova NV, Lovtsius AV, Sapronova OS, Fedotova I, Kolomiets OL, Bogdanov I (2007) Expression and inheritance of a desynaptic phenotype with impaired homologous synapsis in rye. Genetika 43:1424–1433

The International Brachypodium Initiative (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. Nature 463:763–768

Vorontsova M, Shaw P, Reader S, Moore G (2004) Effect of 5-azacytidine and trichostatin A on somatic centromere association in wheat. Genome 47:399–403

Wang TF, Kung WM (2002) Supercomplex formation between Mlh1-Mlh3 and Sgs1-Top3 heterocomplexes in meiotic yeast cells. Biochem Biophys Res Commun 296:949–953

Wang CJ, Carlton PM, Golubovskaya IN, Cande WZ (2009) Interlock formation and coiling of meiotic chromosome axes during synapsis. Genetics 183:905–915

Zickler D, Kleckner N (1998) The leptotene-zygotene transition of meiosis. Annu Rev Genet 32:619–697

Chapter 17
The Secondary Gene Pool of Barley (*Hordeum bulbosum*): Gene Introgression and Homoeologous Recombination

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17.1 Introduction

When applying the gene pool concept (Harlan and de Wet 1971) to the 33 species or subspecies of the genus *Hordeum* listed by Blattner (2009), the primary and secondary gene pools of barley turn out to be composed in a simple manner. Since there are no incompatibility barriers between *Hordeum vulgare* subsp. *vulgare* and its wild progenitor *H. vulgare* subsp. *spontaneum*, these two taxa make up the primary gene pool. The secondary gene pool comprises a single species, *H. bulbosum*, which may be crossed to barley, however with some difficulty. Once hybrids have been obtained, though, the chromosomes of the two different parental genomes may pair in some instances and recombine in a homoeologous fashion. The remaining 31 *Hordeum* species fall into the tertiary gene pool. Hybrids between these species and *H. vulgare* have been achieved in some cases. However, there is no reported success in transferring genes from the tertiary gene pool into barley (Zeller 1998), nor is there any reported evidence for recombination between the parental genomes in those hybrids.

Based on the meiotic pairing of chromosomes in interspecific combinations, the species of the genus *Hordeum* have been assigned to four types of genomes (H, I, Xa, Xu), with *H. vulgare* and *H. bulbosum* belonging to the H-genome species (Blattner 2009). Meanwhile, it has become widely accepted that *H. vulgare* and *H. bulbosum* are the closest related species in the genus *Hordeum* (for an overview, cf. Blattner 2009). Congruently, *H. bulbosum* has remained the only *Hordeum* species not belonging to the primary gene pool, which was shown to allow for homoeologous chromosome pairing and recombination when crossed to cultivated barley.

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Hordeum bulbosum (*Hb*) comprises diploid and tetraploid cytotypes. The diploid cytotype is native to the northern and southern Mediterranean countries as far as Western Greece. Tetraploid cytotypes are found in the east of Greece, Turkey, the Middle East, the Caucasian countries, Turkmenistan, Tajikistan and Afghanistan. *H. bulbosum* has widely been used in cereal breeding for inducing the formation of doubled haploids, because its chromosomes normally are eliminated in the young interspecific hybrid embryos during the first days of development, leaving a single set of the seven chromosomes from the cultivated barley parent which subsequently can be doubled through application of colchicine (Kasha and Kao 1970). In addition to this role in enabling an important breeding method, *H. bulbosum* has long been investigated for its potential as a genetic resource for barley breeding. Since the primary gene pool has been extensively used as a genetic resource to improve disease resistance and other traits in barley, researchers started to turn to the secondary gene pool to dig for novel trait genes not available in *H. vulgare* (*Hv*). A growing number of reports now indicate that *H. bulbosum* carries resistance genes which are of potential interest to barley breeders and which may, indeed, more or less readily be introduced to barley germ plasm via introgression breeding.

17.2 Interspecific Hybrids

According to the widely accepted concept, the primary gene pool is made up of two closely related subspecies rather than different species. Crosses between the two can readily be accomplished and hybrids generally are viable. Consequently, *H. vulgare* subsp. *spontaneum* has long been (and continues to be) a rich resource for introgressing useful and hitherto unexploited trait alleles to cultivated barley. A large number of gene variants for resistance to leaf rust, powdery mildew, scald and the soil-borne virus complex (BaMMV, BaYMV-1, BaYMV-2) as well as for β-amylase activity, respectively, were introduced to barley breeding programmes and mapped to specific chromosomes by the use of molecular markers (Feuerstein et al. 1990; Ivandic et al. 1998; Schüller et al. 1992; Abbott et al. 1992; Ordon et al. 1997; Erkkila 1999; Eglinton et al. 1999). Little information is provided in the literature on the genetic and molecular aspects of recombination between the subspecies' genomes, probably because generally no severe problems are encountered in crossing experiments concerning sterility, introgression rate or linkage drag. Using the *H. vulgare* subsp. *spontaneum* accession Caesarea 26-24 as a donor, barley cv. Harrington as recipient and a total of 47 SSR loci as molecular markers, Matus et al. (2003) observed an average of 12.6 % of introgressed donor genome among 140 BC_2F_6 recombinant chromosome substitution lines (RCSL), which was not significantly different from the expected 12.5 %. Although there also were RCSL with a very low (3 %) or high (30 %) share of introgressed donor genome, this observation is concordant with the assumption of generally undisturbed recombination between the two genomes. The same authors report significant segregation distortions in some chromosome regions, namely, on

chromosomes 1H, 5H and 7H. Referring to the taxonomic consensus of treating the two *Hordeum* forms as subspecies rather than separate species, they conclude that given this consensus, meiotic irregularities, which may manifest themselves in segregation distortion, would not be expected for crosses like the one used in their study. However, a conclusive statement on this item is not possible as long as no differentiation is feasible between the possible genetic causes—namely, meiotic vs. genotypic selection—of the observed segregation distortions.

A more complex situation concerning hybridisation and interspecific recombination with barley is met with the secondary gene pool, which is made up of *H. bulbosum*. In the following, focus is put onto utilising the secondary gene pool in barley breeding.

The first viable, albeit sterile, hybrid was reported by Kuckuck (1934) and resulted from a cross of a diploid *H. vulgare* as the female and a tetraploid *H. bulbosum* as the male parent. Hybrids obtained in early studies usually were not able to produce seeds, and hybrid offspring showing some pollen fertility and anther dehiscence was rare (Konzak et al. 1951). Later on, the yield in interspecific hybrids was substantially improved via embryo rescue (Szigat and Pohler 1982; Pickering 1991; Xu and Kasha 1992). Pickering (1988) obtained several triploid 'VBB' hybrids carrying one set of *H. vulgare* chromosomes (V) and two sets of *H. bulbosum* chromosomes (B). These hybrids possessed fully dehiscent anthers with 45–79 % germinable pollen grains. A tetraploid fertile 'BBVV' hybrid (*H. bulbosum* as female parent) was achieved by Szigat and Pohler (1982).

17.2.1 Homoeologous Recombination

From a breeder's viewpoint, introgressions at homoeologous positions are preferable, such that tailoring of the *H. bulbosum* segments via subsequent rounds of genetic recombination becomes feasible. Meanwhile, there is ample evidence that *H. bulbosum* segments introgressed into barley chromosomes result from homoeologous recombination between the parental genomes. Some of this evidence is outlined in the following.

17.2.1.1 Cytological Evidence

By backcrossing fertile triploid or tetraploid hybrids to barley or through anther culture of hybrids, recombination between the two genomes was achieved, as evidenced by chromosome pairing at meiotic metaphase (Pohler and Szigat 1991; Pickering 1991; Xu and Kasha 1992). For instance, when using the *H. bulbosum*-specific DNA probe pSc119.1 in FISH, Xu and Kasha (1992) detected five *Hb* sub-genomic fragments in a BC1 plant. Using the same probe, Gilpin et al. (1997) detected a *H. bulbosum* segment introgressed into barley chromosome 6HS. Recombination between *H. vulgare* and *H. bulbosum* chromatin was also demonstrated by genomic in situ hybridisation (GISH; Pickering et al. 1997).

17.2.1.2 Evidence from Molecular-Marker Analysis

At the genetic level, evidence for homoeologous recombination comes from genetic mapping studies using molecular markers. For instance, using RFLP markers genetically mapped in barley, Pickering et al. (1995) identified an introgression of a *Hb* segment on chromosome 2HS. Later on, PCR-based anchor markers such as SSR markers simplified the detection of *H. bulbosum* chromatin (Ruge-Wehling et al. 2006). In the majority of cases studied so far, molecular-marker alleles from either parental genome at molecular-marker loci inside or outside the introgressed segment recombine with each other more or less readily (Timmerman et al. 1993; Pickering et al. 1994, 2004, 2006; Ruge et al. 2003; Ruge-Wehling et al. 2006; Scholz et al. 2009), demonstrating that recombination between the two parental genomes is homoeologous.

17.2.1.3 Factors Influencing Homoeologous Recombination

Pickering and Johnston (2005) pointed out that *H. vulgare* × *H. bulbosum* hybrids with consistent and stable chromosome numbers and high intergenomic pairing at meiosis are needed for introgression breeding. In general, though, the number of recombinants obtained from *Hv* × *Hb* crosses has been low, and *H. bulbosum* chromosomes are subject to more or less rapid elimination in interspecific hybrids. There is evidence that both genetic and environmental factors have an influence on the chromosomal behaviour. Ho and Kasha (1975) detected factors on barley chromosomes 2H and 3H that influence the tendency for the elimination of *H. bulbosum* chromosomes in interspecific hybrids. Temperatures of <17.5 °C during the first few days after pollination were found favourable for the formation of diploid interspecific hybrids (Pickering 1985). Stability of *Hb* chromosomes in amphidiploid VVB hybrids was reported to depend on the genotype of both the *Hv* and *Hb* parents, and with regard to introgression breeding, use of parent lines selected for high and stable chromosome pairing was proposed (Thomas and Pickering 1983, 1985). As to the influence of the *Hv* parent, one or several dominant genes were suggested to be present in the barley cv. 'Vada' which prevented the elimination of *Hb* chromosomes from the amphidiploid VVB hybrid (Thomas and Pickering 1983). Using genomic in situ hybridisation (GISH), Zhang et al. (1999) demonstrated that recombination between the different parental chromosomes involved only distal chromosome regions. Furthermore, they observed significant differences between two diploid *H. vulgare* × *H. bulbosum* hybrids with regard to chromosome stability (i.e. retention of *H. bulbosum* chromosomes) and recombination frequency. Interestingly and for unknown reasons, while one hybrid led to significantly higher recombination frequency as compared with the second hybrid, the latter one had a ratio of recombination to meiotic pairing frequency, which was almost twice as high as in the former one. In both hybrids, meiotic pairing frequency greatly exceeded recombination. In summary, the results of Zhang et al. (1999) and others suggest that recombination frequency is genetically influenced and hybrid parents may be selected for optimising the yield of recombinants.

17.2.2 Introgression Sites and Sizes

Hordeum bulbosum introgressions are generally found at distal positions of barley chromosomes. Barley chromosomal arms greatly differ in the frequencies with which introgressions occur. Pickering et al. (2004) reported that mostly the chromosome arms 2HL and 4HL were found to carry *H. bulbosum* segments. In contrast, few introgressions were observed on chromosomes 1HS, 3HS, 3HL, 4HS and 5HS. Combining the results of cytological and molecular-marker-based analyses, it can be stated that meanwhile, *H. bulbosum* introgressions have been achieved on all the 14 chromosomal arms of barley (Pickering et al. 2004; Johnston et al. 2009; Scholz et al. 2009), thus making a large proportion of the *H. bulbosum* genome accessible to introgression breeding in barley.

Hordeum bulbosum sub-chromosomal segments introgressed into barley differ in their size. The two largest introgressions observed so far had approximately half the physical size of the long arms of chromosomes 4H and 6H, respectively (Pickering et al. 2004). Introgressions of comparably small sizes of 1.8 cM and 3.6 cM were reported by Ruge et al. (2003) and Pickering et al. (2006) on chromosome 6HS and 4HS, respectively.

With regard to plant breeding, though, accessibility of an introgressed *Hb* segment to size reduction via subsequent rounds of recombination appears more relevant than its initial size observed in the interspecific hybrid. Compared with the situation in a pure barley-genetic background, the relative ease with which recombination occurs in heterozygous introgression genotypes may vary between introgressions as well as along a given introgressed segment. In many cases, recombination frequencies were found to be similar or moderately suppressed relative to the pure-barley situation. But there are exceptions. This aspect is further explained below.

17.3 Transfer of Disease Resistance and Marker-Assisted Selection

During the past 20 years, it has become evident that *H. bulbosum* provides a rich source of resistances to a variety of pathogens, including fungal as well as viral diseases.

17.3.1 Fungus Resistance

Xu and Snape (1989) screened two tetraploid and two diploid *H. bulbosum* accessions with five isolates each of powdery mildew, yellow rust and leaf rust. All accessions proved to be immune to powdery mildew (PM), and the tetraploid accessions were also resistant to yellow rust (*Puccinia striiformis*) and leaf rust (*P. hordei*) isolates. Diploid and triploid hybrids of these accessions and susceptible barley parents were resistant to PM, and the single tested triploid hybrid also expressed resistance to

yellow rust. Xu and Kasha (1992) produced backcross progeny (BC1) of resistant triploid *Hv* × *Hb* hybrids and demonstrated that PM resistance could be transferred from the wild species to barley as a single dominant resistance factor, thereby opening up the perspective of using *H. bulbosum* as resistance resource in plant breeding. Using GISH and RFLP analysis, Kasha et al. (1996) showed that this PM-resistance gene had been transferred to the 2HL chromosome arm in barley.

Another dominant PM-resistance gene was described by Pickering et al. (1987, 1995) and Michel (1996) in segregating F2 offspring obtained from a selfed tetraploid hybrid that was derived from a cross of tetraploid *H. vulgare* and tetraploid *H. bulbosum*. This PM-resistance gene was located to barley chromosome 2HS by the use of the barley RFLP anchor marker cMWG862 (Pickering et al. 1995) and was found to be inherited together with resistance to leaf rust, which could be explained by both genes being located on the same introgressed *H. bulbosum* sub-genomic fragment on barley chromosome 2HS (Pickering et al. 1998). Evidence was presented by the authors that the two genes may be separated via recombination and, hence, the original *Hb* introgression may be reduced in its size. Ruge et al. (2004) came to the same conclusion when analysing a 2HS introgression derived from a differing *Hb* cross parent. Again, two resistance genes to powdery mildew and leaf rust, namely Ml^{Hb} and $Rph20^{Hb}$, respectively, had jointly been transferred to barley; however, they could be separated from each other via recombination.

Taken together, there is ample evidence that the secondary gene pool holds a variety of resistances to fungal diseases in barley, among them powdery mildew, leaf rust, yellow rust, stem rust, *Rhynchosporium secalis*, *Septoria passerinii* and *Typhula incarnata*. A summary of introgressed resistance genes is given in Table 17.1. The majority of resistances described so far are dominantly expressed, with one exception concerning resistance to stem rust which was reported by Fetch et al. (2009) to be inherited as a recessive trait (Table 17.1).

In some cases, resistance genes introgressed from *H. bulbosum* into barley represent novel genes, which reside at loci different from those known from the primary gene pool. This is the case for the two PM-resistance factors reported by Pickering et al. (1995) and Michel (1996). Whereas these factors seem to be allelic or else closely linked to each other, they are nonallelic to PM-resistance genes drawn from the primary gene pool. Even when independently derived introgressions turn out to carry allelic resistances, each of these may contribute to broaden the genetic basis for disease resistance. Since *H. bulbosum* is strictly outcrossing, populations and individuals are expected to be genetically heterogeneous or heterozygous; there is a chance that introgressions carrying allelic resistance genes will introduce alleles, which react differently to races or pathotypes in barley. This was demonstrated for PM resistance. Michel (1996) showed that the two independent albeit allelic introgressions mentioned above represent different alleles, which react differently to a set of PM isolates. Moreover, when combined in F1, the two introgressions complemented each other to give a more robust, immune-like resistance to a variety of PM isolates as compared to the reaction of each single introgression (Michel 1996). Independent introgressions from differing *Hb* parents have been provided for resistance to powdery mildew (Xu and Kasha 1992; Pickering et al. 1995; Michel 1996), leaf rust (Pickering et al. 1998; Ruge et al. 2004), stem rust (Pickering

Table 17.1 Resistances to fungal or viral diseases in barley introgressed from *H. bulbosum*

Resistance to pathogen	Gene designator	Introgressed on barley chromosome	References
Erysiphe graminis	–	2HL	Kasha et al. (1996)
	–	2HL	Pickering et al. (1995)
	–	2HL	Michel (1996)
	–	2HS	Pickering et al. (1998)
	–	7HL	Pickering et al. (2004)
	Ml^{Hb}	2HS	Ruge et al. (2004)
Puccinia hordei	–	1HL	Pickering et al. (2004)
	–	2HS	Pickering et al. (1998)
	$Rph20^{Hb}$	2HS	Ruge et al. (2004)
	$Rph21^{Hb}$	2HL	Ruge et al. (2004)
	$Rph22^{Hb}$	5HL	Ruge et al. (2004)
Puccinia graminis	*Rpg6*	6HS	Fetch et al. (2009)
	–	7HL	Pickering et al. (2004)
Septoria passerinii	–	4HL	Toubia-Rahme et al. (2003)
Rhynchosporium secalis	$Rrs16^{Hb}$	4HS	Pickering et al. (2006)
BaMMV, BaYMV-1, -2	$Rym14^{Hb}$	6HS	Ruge et al. (2003)
	$Rym16^{Hb}$	2HL	Ruge-Wehling et al. (2006)
BYDV	$Ryd4^{Hb}$	3HL	Scholz et al. (2009)

et al. 2004; Fetch et al. 2009) and the soil-borne virus complex (BaMMV, BaYMV-1, BaYMV-2) (Ruge et al. 2003; Ruge-Wehling et al. 2006).

17.3.2 Virus Resistance

The secondary gene pool has proven a valuable source also in respect to virus resistance. Michel (1996) provided evidence for two dominant barley mild mosaic virus (BaMMV)-resistance genes that had been transferred from a tetraploid *H. bulbosum* accession to barley. Later on, these two genes were designated as $Rym14^{Hb}$ and $Rym16^{Hb}$, mapped relative to molecular markers and assigned to barley chromosomes 6HS and 2HL, respectively, using FISH and molecular anchor markers (Ruge et al. 2003; Ruge-Wehling et al. 2006). $Rym14^{Hb}$ was found to cosegregate among 168 individuals with two RFLP anchor markers and one codominant STS marker, *Xiac500*(*STS*). This marker had been derived from a differential cDNA analysis of two bulks made of resistant vs. susceptible individuals, respectively, of a segregating F5 mapping population. From this analysis, a cDNA-AFLP fragment was obtained which was detectable only in the resistant bulk and only following BaMMV inoculation. Regarding this specific origin as well as the cosegregation with

BaMMV resistance, *Xiac500*(*STS*) potentially provides a diagnostic marker for marker-assisted selection of *Rym14*^{Hb} carriers in plant breeding programmes.

Among the BaMMV-resistance genes described in barley so far, *Rym14*^{Hb} and *Rym16*^{Hb} are unique in that they are dominantly expressed. All other BaMMV resistances derived from the primary gene pool are known to be recessive. This poses the question as to the biological resistance mechanism underlying the two genes. The question still remains to be answered. As pointed out by Ruge-Wehling et al. (2006), the resistance is effective following mechanical inoculation with BaMMV, which suggests that post-transmission steps are influenced by each of the two genes. In any case, while other genes, i.e. *rym3*, *rym5* and *rym6* (Kanyuka et al. 2004), have been overcome by novel virus strains, *Rym14*^{Hb} and *Rym16*^{Hb} appear to be more durable in their effectiveness (Habekuß et al. 2005).

Another dominant resistance introgressed from the secondary gene pool is effective against barley yellow dwarf virus (BYDV), a disease of growing economic importance in many regions where winter barley is cultivated. In contrast to the BaMMV/BaYMV virus complex, which is transmitted by the soil-borne fungus *Polymyxa graminis*, BYDV is transmitted by aphids (*Rhopalosiphum padi*, *Sitobion avenae*). A BYDV resistance was introgressed from a tetraploid *H. bulbosum* accession to the susceptible barley cv. 'Igri'. Resistance was reported to be inherited as a monogenic dominant trait and was assigned to a novel resistance gene, *Ryd4*^{Hb}. In plants homozygous or heterozygous for *Ryd4*^{Hb}, this gene confers immunity against the strain BYDV-PAV around Aschersleben, as demonstrated by ELISA values of zero or close to zero. Using cytogenetic detection methods (FISH, GISH) as well as molecular anchor markers, *Ryd4*^{Hb} was assigned to barley chromosome 3HL (Scholz et al. 2009). Due to its immune-like mode of action, *Ryd4*^{Hb} is of potential interest to barley breeders and farmers, because plants carrying this gene are expected not only to be tolerant in terms of BYDV-mediated yield reduction. Rather, *Ryd4*^{Hb} carriers are expected to have nonhost properties to the virus and thus would not support virus loading and spreading by aphid vectors. However, before a practical use of *Ryd4*^{Hb} in plant breeding programmes can be launched, some additional work will have to be done to tailor the introgressed segment, as outlined in the following section, and to devise tools for marker-assisted selection (MAS) of suitable *Ryd4*^{Hb} carriers in the breeder's nursery. MAS is especially rewarding in the case of virus resistance because direct assessment of this trait is expensive to do.

17.3.3 *Genetic Tailoring of* Hb *Introgressions in Barley Germ Plasm*

An introgressed segment carrying a valuable trait gene from the secondary gene pool into barley may be quite large (if defined in size at all) in its original version and, thus, be of doubtful value for a plant breeder. Usually, the introgression has to be reduced in size by substituting *Hv* chromatin for most of the *Hb* chromatin, while retaining the valuable trait gene. This is done by a marker-assisted search for

recombinants among selfed or backcrossed offspring. To find suitable recombinants may be accomplished with more or less ease, depending on the degree of homoeologous chromosomal pairing, and hence the probability for genetic recombination of *Hb* and *Hv* chromatin along the introgressed sub-chromosomal segment. The final proof whether or not an introgressed segment has been sufficiently downsized must, of course, come from comparative yield trials which usually are performed under 'common practice', in the absence of severe infection pressure.

An estimate of how much the recombination activity of a homoeologous pair of chromosomes is altered due to the existence of an introgressed *Hb* sub-chromosomal segment may be obtained by mapping molecular markers along the introgressed segment and comparing their distances with a consensus map set up in a pure barley-genetic background.

For instance, the *Hb* introgression reported to harbour the scald-resistance gene $Rrs16^{Hb}$ (Pickering et al. 2006) was estimated 3.6 cM in size, as judged from the genetic distance of the most distant molecular anchor markers which still segregated with a *Hb* allele. In comparison, the same marker interval extends over 5 cM in a barley consensus map, suggesting that in this case, the introgression is relatively small and there is little, if any, suppression/reduction of recombination along the introgressed segment. Thus, further downsizing of the introgression should readily be accomplished via several rounds of recombination. Molecular markers flanking $Rrs16^{Hb}$ in 0.1 cM and 0.3 cM distance, respectively, are available for starting a marker-assisted pre-breeding project.

In the case of $Rym16^{Hb}$ (Ruge-Wehling et al. 2006), the introgressed *Hb* sub-chromosomal segment extended over 30 cM on the long arm of barley chromosome 2H, with $Rym16^{Hb}$ mapping at the distal end of the introgression. At its proximal end, the introgressed segment had a region of approx. 7 cM in size, which corresponded to approx. 28 cM in a barley consensus map, indicating that recombination of *Hb* with *Hv* chromatin was suppressed in this section by a factor of 4. This proximal part carried a lethality factor which prevented the formation of viable homozygous-resistant offspring. A larger, distal part (20.4 cM) of the introgressed segment appeared not to be subject to pronounced recombination suppression/reduction, since the extension of the respective molecular-marker interval compared to 18 cM in a pure-barley map. As a consequence, in a first round of marker-assisted selection, recombinant resistant offspring could readily be identified which was devoid of the proximal part of the original introgression.

In the case of $Rym14^{Hb}$, size reduction will probably be more time-consuming since the introgressed segment corresponds to a 13-cM marker interval in the consensus map [equivalent to 21 Mb in the physical map by Künzel et al. (2000)], while in segregating offspring from a $Hv \times Hb$ hybrid, its genetic size is only 1.8 cM, which means a suppression/reduction of recombination by a factor of 7 (Ruge et al. 2003).

Association of an introgressed trait gene with otherwise negative effects may pose a challenge to pre-breeding. This is exemplified by the $Ryd4^{Hb}$ introgression (Scholz et al. 2009), which was found to bring about a number of negative side effects. Firstly, a segregation-distorting locus (SDL) was linked to resistance, leading to selection against gametes carrying the *Hb* introgression and, hence,

preventing the formation of homozygous-resistant offspring. While this SDL could finally be separated from $Ryd4^{Hb}$ via recombination, a recessive sublethality factor remained linked to the resistance. This factor leads to severe growth depression of homozygous-resistant plants, thus preventing their use in a plant breeding programme at present. Additional molecular markers will have to be developed to perform a high-resolution mapping of the residual introgressed segment and to use this information in a marker-assisted pre-breeding programme.

Conclusion

The past 20 years has seen a major breakthrough in the utilisation of plant genetic resources for barley breeding. In 1991, von Bothmer and Jacobsen came to the conclusion that 'the plant breeding potential for improvement of barley using wide hybridization is, at the present state of knowledge, to utilize the gene pool available within *H. vulgare*, i.e. landraces, wild and weedy form of subsp. *spontaneum*, where no sterility barriers are operating in combinations with cultivated barley'. Since then, a number of reports have been published on the successful introgression of *H. bulbosum* genes into barley, their usefulness as novel trait genes in barley breeding and the tailoring of introgressed segments via marker-assisted selection. Today we can be confident in stating that the secondary gene pool of cultivated barley has been made accessible to the plant breeder as a novel resource for enriching the genetic diversity of the barley germ plasm. For obvious reasons, monogenic disease resistances were among the first traits improved in barley by using the secondary gene pool. At least some of these disease resistances (e.g. the BYDV resistance mediated by $Ryd4_{Hb}$) seem to be controlled by genes different from those described so far in the primary gene pool and are expected to confer relatively durable resistance. What remains to be done is to evaluate the secondary gene pool with respect to other possibly quantitative traits like tolerance to abiotic stresses such as drought, cold and salinity.

The future prospects of approaching the secondary gene pool in a more systematic way will depend on the progress that can be achieved in two major directions. Firstly, the protocols for generating fertile interspecific $Hv \times Hb$ hybrids in sufficiently high frequency and for deliberately achieving introgressions in cultivated barley will have to be further optimised and refined. Secondly, genomic tools will have to be used for a more efficient genetic tailoring of *H. bulbosum* introgressions. Such tools will make use of the tremendous gain of knowledge achieved with regard to genome orthology among the grasses including rice, to the sequencing of candidate genes in grasses and to constructing a 'genome zipper' which exactly predicts the position of genes and markers in barley and related genomes (Mayer et al. 2011). As a result, optimised species hybridisation, marker-assisted size tuning of introgressed segments and selection markers diagnostic for the gene of interest will make the utilisation of the secondary gene pool a less random and more predictable approach.

References

Abbott DC, Brown AHD, Burdon JJ (1992) Genes for scald resistance from wild barley (*Hordeum vulg*are ssp. *spontaneum*) and their linkage to isozyme markers. Euphytica 61:225–231

Blattner F (2009) Progress in phylogenetic analysis and a new infrageneric classification of the barley genus *Hordeum* (Poaceae: Triticeae). Breed Sci 59:471–480

Eglinton JK, Evans DE, Brown AHD, Langridge P, McDonald G, Jefferies SP, Barr AR (1999) The use of wild barley (*Hordeum vulgare* ssp. *spontaneum*) in breeding for quality and adaptation. In: Proceedings of 9th Australian barley technical symposium 2.29, pp 1–6

Erkkila MJ (1999) Intron III-specific markers for screening of β-amylase alleles in barley cultivars. Plant Mol Biol Rep 17:139–147

Fetch T Jr, Johnston PA, Pickering R (2009) Chromosomal location and inheritance of stem rust resistance transferred from *Hordeum bulbosum* into cultivated barley (*H. vulgare*). Phyto 99:339–343

Feuerstein U, Brown AHD, Burdon JJ (1990) Linkage of rust resistance genes from wild barley (*Hordeum spontaneum*) with isozyme markers. Plant Breed 104:318–324

Gilpin MJ, Pickering RA, Fautrier AG, McNeil DL, Szigat G, Hill AM, Kynast RG (1997) Morphological and molecular analysis of androgenetic, selfed and backcrossed plants produced from a *Hordeum vulgare* x *H. bulbosum* hybrid. Plant Breed 116:505–510

Habekuß A, Kühne T, Rabenstein F, Krämer I, Ehrig F, Ruge-Wehling B, Huth, W, Ordon F (2005) Detection of an *rym5* resistance breaking virus strain in Germany. In: 6th symposium international working group on plant viruses with fungal vectors, Bologna, Italy, 5–7 Sept 2005, p 60

Harlan JR, de Wet JMJ (1971) On the origin of barley: a second look. In: Nilan RA (ed) Barley genetics II. Proceedings of 2nd barley genetics symposium. Washington State University Press, Pullman, pp 45–50

Ho KM, Kasha KJ (1975) Genetic control of chromosome elimination during haploid formation in barley. Genetics 81:263–275

Ivandic V, Walther U, Graner A (1998) Molecular mapping of a new gene in wild barley conferring complete resistance to leaf rust (*Puccinia hordei* Otth). Theor Appl Genet 97:1235–1239

Johnston PA, Timmerman-Vaughan GM, Farnden KJF, Pickering R (2009) Marker development and characterisation of *Hordeum bulbosum* introgression lines: a resource for barley improvement. Theor Appl Genet 118:1429–1437

Kanyuka K, Mc Grann G, Alhudaib K, Hariri D, Adams MJ (2004) Biological and sequence analysis of a novel European isolate of *Barley mild mosaic virus* that overcomes the barley *rym5* resistance gene. Arch Virol 149:1469–1480

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*H. vulgare* L.). Nature 225:874–876

Kasha KJ, Pickering RA, William HM, Hill A, Oro R, Reader S, Snape JW (1996) GISH and RFLP facilitated identification of a barley chromosome carrying powdery mildew resistance from Hordeum bulbosum. In: Proceedings of the 7th barley genetics symposium, vol 1. University Extension Press, University of Saskatchewan, pp 338–340

Konzak CF, Randolf LF, Jensen NF (1951) Embryo culture of barley species hybrids. J Hered 42:125–134

Kuckuck H (1934) Artkreuzungen bei Gerste. Züchter 6:270–273

Künzel G, Korzun L, Meister A (2000) Cytologically integrated physical restriction fragment-length polymorphism maps for the barley genome based on translocation breakpoints. Genetics 154:397–412

Matus I, Corey A, Filichkin T, Hayes PM, Vales MI, Kling J, Riera-Lizarazu O, Sato K, Powell W, Waugh R (2003) Development and characterization of recombinant chromosome substitution lines (RCSLs) using *Hordeum vulgare* subsp. *spontaneum* as a source of donor alleles in a *Hordeum vulgare* subsp. *vulgare* background. Genome 46:1010–1023

Mayer KF, Martis M, Hedley PE, Šimkova H, Liu H, Morris JA, Steuernagel B, Taudien S, Roessner S, Gundlach H, Kubalákova M, Suchánková P, Murat F, Felder M, Nussbaumer T, Graner A, Salse J, Endo T, Sakai H, Tanaka T, Itoh T, Sato K, Platzer M, Matsumoto T, Scholz U, Doležel J, Waugh R, Stein N (2011) Unlocking the barley genome by chromosomal and comparative genomics. Plant Cell 23:1249–1263

Michel M (1996) Untersuchungen zur Übertragung von Resistenzgenen aus der Wildart *Hordeum bulbosum* L. in die Kulturgerste *Hordeum vulgare* L. PhD Thesis, Lehrstuhl für Pflanzenbau und Pflanzenzüchtung, Technische Universität München, Germany

Ordon F, Schiemann A, Friedt W (1997) Assessment of the genetic relatedness of barley accessions (*Hordeum vulgare s.l.*) resistant to soil-borne mosaic-inducing viruses (BaMMV, BaYMV, BaYMV-2) using RAPDs. Theor Appl Genet 94:325–330

Pickering RA (1985) Partial control of chromosome elimination by temperature in immature embryos of *H. vulgare* L. x *H. bulbosum* L. Euphytica 34:869–874

Pickering RA (1988) The production of fertile triploid hybrids between *Hordeum vulgare* L. (2n = 2x =14) and *H. bulbosum* L. (2n = 4x = 28). Barley Genet Newsl 18:25–29

Pickering RA (1991) The production of fertile triploid hybrids from crosses between *Hordeum vulgare* L. (2n = 4x =28) and *H. bulbosum* L. (2n = 2x = 14). Hereditas 114:227–236

Pickering R, Johnston PA (2005) Recent progress in barley improvement using wild species of *Hordeum*. Cytogenet Genome Res 109:344–349

Pickering RA, Rennie WF, Cromez MG (1987) Disease resistance material available from the wide hybridization programme at DSIR. Barley Genet Newsl 31:248–250

Pickering RA, Timmermann GM, Cromey MG, Melz G (1994) Characterisation of progeny from backcrosses of triploid hybrids between *Hordeum vulgare* L. (2x) and *H. bulbosum* L. (4x) to *H. vulgare*. Theor Appl Genet 88:460–464

Pickering RA, Hill AM, Michel M, Timmerman-Vaughan GM (1995) The transfer of a powdery mildew resistance gene from *Hordeum bulbosum* L. to barley (*H. vulgare* L.) chromosome 2 (2I). Theor Appl Genet 91:1288–1292

Pickering RA, Hill AM, Kynast RG (1997) Characterization by RFLP analysis and genomic *in situ* hybridization of a recombinant and a monosomic substitution plant derived from *Hordeum vulgare* L. x *Hordeum bulbosum* L. crosses. Genome 40:195–200

Pickering RA, Steffenson BJ, Hill AM, Borovka I (1998) Association of leaf rust and powdery mildew resistance in a recombinant derived from a *Hordeum vulgare* x *Hordeum bulbosum* hybrid. Plant Breed 117:83–84

Pickering R, Johnston PA, Ruge B (2004) Importance of the secondary genepool in barley genetics and breeding. I. Cytogenetics and molecular analysis. Czech J Genet Plant Breed 40:73–78

Pickering R, Ruge-Wehling B, Johnston PA, Schweizer G, Ackermann P, Wehling P (2006) The transfer of a gene conferring resistance to scald (*Rhynchosporium secalis*) from *Hordeum bulbosum* into *H. vulgare* chromosome 4HS. Plant Breed 5:576–579

Pohler W, Szigat G (1991) Somatic *Hordeum vulgare* chromosome elimination in *H. vulgare* – *H. bulbosum* hybrids as revealed by the occurrence of alloplasmic *H. bulbosum* sectors. Biol Zentralbl 110:42–51

Ruge B, Linz A, Pickering R, Proesler G, Greif P, Wehling P (2003) Mapping of *Rym14*^{Hb}, a gene introgressed from *Hordeum bulbosum* and conferring resistance to BaMMV and BaYMV in barley. Theor Appl Genet 107:965–971

Ruge B, Linz A, Habekuß A, Flath K, Wehling P (2004) Introgression and mapping of novel resistance genes from the secondary genepool of barley – *Hordeum bulbosum*. Proceedings of 9th international barley genetics symposium, Part II, 20–26 June 2004, pp 729–736

Ruge-Wehling B, Linz A, Habekuß A, Wehling P (2006) Mapping of *Rym16*^{Hb}, the second soil-borne virus-resistance gene introgressed from *Hordeum bulbosum*. Theor Appl Genet 113:867–873

Scholz M, Ruge-Wehling B, Habekuß A, Schrader O, Pendinen G, Fischer K, Wehling P (2009) *Ryd4*^{Hb}: a novel resistance gene introgressed from *Hordeum bulbosum* into barley and

conferring complete and dominant resistance to the barley yellow dwarf virus. Theor Appl Genet 119:837–849

Schüller C, Backes G, Fischbeck G, Jahoor A (1992) RFLP markers to identify the alleles on the MLA locus conferring powdery mildew resistance in barley. Theor Appl Genet 84:330–338

Szigat G, Pohler W (1982) *Hordeum bulbosum* x *H. vulgare* hybrids and their backcrosses with cultivated barley. Cereal Res Commun 10:73–78

Thomas HM, Pickering RA (1983) Chromosome elimination in *Hordeum vulgare* x *H. bulbosum* hybrids. I. Comparisons of stable and unstable amphidiploids. Theor Appl Genet 66:135–140

Thomas HM, Pickering RA (1985) The influence of parental genotype on the chromosome behaviour of *Hordeum vulgare* x *H. bulbosum* diploid hybrids. Theor Appl Genet 71:437–442

Timmerman GM, Pickering RA, Melz G (1993) Characterization of *Hordeum vulgare – Hordeum bulbosum* chromosome substitution lines by restriction fragment length polymorphism analysis. Genome 36:507–511

Toubia-Rahme H, Johnston PA, Pickering RA, Steffenson BJ (2003) Inheritance and chromosomal location of *Septoria passerinii* resistance introgressed from *Hordeum bulbosum* into *Hordeum vulgare*. Plant Breed 122:405–409

von Bothmer R, Jacobsen N (1991) Interspecific hybrids within the genus *Hordeum*. In: Gupta PK, Tsuchiya T (eds) Chromosome engineering in plants: genetics, breeding, evolution. Part A. Elsevier, Amsterdam, pp 411–431

Xu J, Kasha KJ (1992) Transfer of a dominant gene for powdery mildew resistance and DNA from *Hordeum bulbosum* into cultivated barley (*H. vulgare*). Theor Appl Genet 84:771–777

Xu J, Snape JW (1989) The resistance of *Hordeum bulbosum* and its hybrids with *H. vulgare* to common fungal pathogens. Euphytica 41:273–276

Zeller FJ (1998) Nutzung des genetischen Potentials der *Hordeum*-Wildarten zur Verbesserung der Kulturgerste (*Hordeum vulgare* L). J Appl Bot 72:162–167

Zhang L, Pickering R, Murray B (1999) Direct measurement of recombination frequency in interspecific hybrids between *Hordeum vulgare* and *H. bulbosum* using genomic *in situ* hybridization. Heredity 83:304–309

Chapter 18
Genome-Wide Association Scans (GWAS)

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18.1 Introduction

Genetic analysis in barley using molecular markers has been conducted extensively over the past 20 years. Based initially on the framework provided by the development of genome-wide linkage maps (Graner et al. 1990; Kleinhofs et al. 1993), important major genes and quantitative trait loci (QTL) have been located using a range of F2, RIL and doubled haploid mapping populations. These studies have yielded genetic markers that have been used extensively for the indirect selection of traits that are difficult to assess in a breeding programme context [e.g. resistance to the soilborne pathogen barley yellow mosaic virus (BaYMV) (Graner et al. 1998) and epiheterodendrin content in barley for the whisky industry (Thomas 2003)] and that, if translated into financial value, have generated millions of € by increasing yield under adverse conditions or improving product quality. These same studies have led to the identification of causal genes and corresponding alleles that confer a variety of traits, generally through the well-established route of positional cloning

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[e.g. mlo (Büschges et al. 1997), Mla (Wei et al. 1999), Rym4/Rym5 (Stein et al. 2005), Vrn3 (Yan et al. 2006), Ppd-H1 (Turner et al. 2005)].

The use of experimental mapping populations derived from parents that contrast for a target trait has however been of limited use to the more applied research sector because the parents used are frequently irrelevant to current breeding germplasm and the traits identified are already frequently fixed in the elite breeding gene pool. Consequently a move to assess traits that still segregate in such much more closely related germplasm has been promoted. Genome-wide association scans (GWAS) provide a mechanism to assess variation that segregates in a gene pool, rather than in a biparental population. Fashioned originally in human genetics where it was developed to take account of the types of populations available for genetic analysis, it has become popular in plant genetic research over the last decade (Waugh et al. 2009). GWAS is attractive for multiple reasons, the first of which is that it potentially provides an opportunity to exploit existing and extensive phenotypic data collected during the plant registration process, thus making it directly relevant to current breeding material. Second, it holds the promise of increasing genetic resolution because GWAS populations typically contain more genetic breakpoints and more alleles than are found in conventional mapping populations. However, GWAS approaches also raise issues in genetic analysis. These are largely caused by the origins and history of the population, which introduce a tendency to reveal significant false-positive associations due to factors other than genetic linkage. Here, we will attempt to summarise some of the progress and the problems that have been encountered in establishing effective GWAS in barley and the approaches that have been developed or applied to take account of them. Whilst several studies from various groups have shown that GWAS in barley can be an effective tool for QTL analysis, within our group we have focused on the potential of the approach for identifying the actual genes underlying specific plant phenotypes.

18.2 Linkage Disequilibrium in Different Barley Gene Pools

Determining the extent of linkage disequilibrium (LD) in a target gene pool allows us to estimate the number of molecular markers required to conduct a saturated GWAS and the mapping resolution it is likely to achieve. Studies in outbreeding (e.g. maize; Remington et al. 2001) and inbreeding (e.g. *Arabidopsis*; Nordborg et al. 2002) species revealed that the extent of LD is very different according to breeding habit and, as predicted theoretically, tends to be considerably less extensive in outbreeders. For inbreeders, the derived homozygosity reduces the effective recombination rate at each round of meiosis, and LD is more extensive. However, LD is also highly population dependent, as reported for several species including barley (Caldwell et al. 2006). This has led subsequently to significantly revised estimates of LD (Nordborg et al. 2005; Kim et al. 2007; Yan et al. 2009). Thus, in barley, whilst the initial studies of Kraakman et al. (2004) assayed a collection of 146 modern two-row spring barley cultivars using 236 AFLPs observed significant

LD between markers extending up to 10 cM, Morrell et al. (2005) concluded that intra-locus LD decayed at a rate similar to that observed in outbreeding maize from looking at intra- and inter-gene LD in 18 nuclear genes in a collection of 25 wild barley accessions sampled from across its natural geographic range.

Caldwell et al. (2006) illustrated this population dependency issue very clearly. By resequencing genes present on a small BAC contig across cultivars, landraces and wild barley isolates, they observed a sharp decline in the extent of LD with increasing wildness, consistent with the evolutionary time between the individuals within each sampled set. Although this study was based on a small region of the barley genome, its general conclusions have been confirmed several times since in both diverse and narrow barley collections, and more importantly at a genome-wide scale (Malysheva-Otto et al. 2006; Cuesta-Marcos et al. 2010; Zhang et al. 2009). Subsequent studies have also shown that LD based on physical distance measurements varies enormously according to genomic position (Hamblin et al. 2010; Comadran et al. 2011a). Thus, within the same elite-cultivated gene pool, LD may extend from hundreds of kilobases in recombinogenic portions of the genome to hundreds of megabases in the rarely recombining (but gene rich) centromere-proximal regions.

18.3 Genetic Markers

A knowledge and understanding of how LD is elaborated in different gene pools allows us to estimate the number of genetic markers required to best capture the diversity and recombination history of the population (Fig. 18.1). In the cultivated gene pool where LD is extensive, a relatively small number of markers are theoretically required to capture the majority of the recombination events present

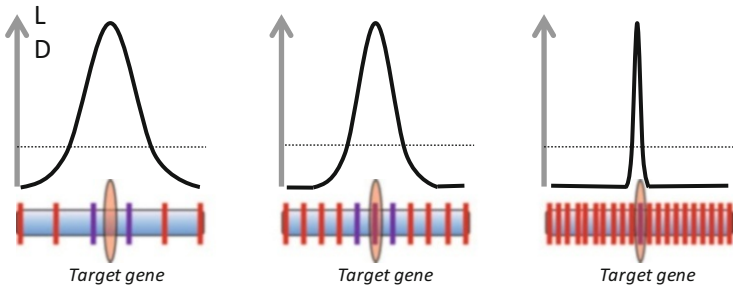

Fig. 18.1 A cartoon of linkage disequilibrium (LD) in three different barley gene pools (*black arc*). The three graphs from *left* to *right* symbolise the situation observed in the cultivated, landrace and wild gene pools of barley. They show the impact of changes in the extent in LD due to recombination on the number of genetic markers (*vertical red* and *purple lines* on a portion of a hypothetical chromosome) required to detect significant associations between a marker and a target gene. Thus, with more extensive LD in the cultivated gene pool, fewer markers are required to detect the target gene by GWAS when compared to the wild gene pool

in the population. Based on practical observations, this led Rostoks et al. (2006) to suggest that roughly 5×10^2 to 5×10^3 markers may be required to adequately survey the elite NW European barley gene pool. At the other end of the spectrum, the number required to capture the resolution afforded by thousands of years of effective recombination in wild species are likely to exceed this by orders of magnitude. In many respects the density of markers required has constrained the adoption of GWAS, and in many species there is still insufficient understanding of LD and available genetic markers and marker technologies that can be adequately applied for this purpose.

Genetic marker technologies have been evolving continuously for the last 25 years or more in barley, as in most major crops. Despite the early attempts by Kraakman et al. (2004, 2006) and Kraakman (2005) to apply AFLP technology to association mapping in barley, it only became realistic to attempt GWAS studies in large populations of related germplasm with the availability of high-throughput (HTP) single-nucleotide polymorphism (SNP) marker technologies such as Illumina's 'GoldenGate' oligo pool assays (OPAs) (Fan et al. 2003; Rostoks et al. 2005, 2006; Close et al. 2009). These technologies effectively eradicated unintentional error within genotypes introduced during serial marker assays and allowed the collection of massive marker datasets that were virtually inconceivable only a few years earlier. These markers also revealed much about legacy biparental mapping populations, highlighting genotypic errors and unintentional mix-ups, sometimes at frequencies of 10 % or higher, and by eradicating single-marker double recombinants, promoted map shrinkage to lengths broadly consistent with observed numbers of chiasmata during meiosis (Nilsson et al. 1993). HTP SNP marker sets were similarly informative in germplasm collections, revealing sample incongruence, heterogeneity and duplication at previously unprecedented resolution. Recently, SNP platforms containing many thousands of markers have been developed, such as Illumina's Barley-OPA1 (BOPA1), Barley-OPA2 (BOPA2) (Close et al. 2009) and iSELECT platforms (Comadran et al. 2012), and used widely to genotype thousands of samples in both the public and private sectors (e.g. AGOUEB, http://www.agoueb.org; BarleyCAP, http://barleycap.cfans.umn.edu; ExBarDiv: http://pgrc.ipk-gatersleben.de/barleynet/projects_exbardiv.php) (Waugh et al. 2010).

Despite their success, these SNP marker platforms are already coming under threat from methods that exploit the massive increase in data volumes and reduction in costs associated with next-generation sequencing technologies (NGS). Methods including the use of reduced-representation libraries (RRLs), complexity reduction of polymorphic sequences (CRoPS^{TM}), restriction-site-associated DNA sequencing (RAD-seq) and low-coverage genotyping by sequencing (GbS) provide ultra-high density genotyping at extremely low cost per datapoint (reviewed in Davey et al. 2011). These sequence-based methods have no prior development requirements and can be used in species lacking reference genome sequences. In barley RAD-seq on the Oregon Wolfe Barley population generated 463 new RAD loci on all seven linkage groups (Chutimanitsakun et al. 2011) and GbS on the same population over 25,000 additional markers at exceedingly low cost (Elshire et al. 2011). However, at this point in time, the commercial propositions such as the iSELECT platform remain more accessible to the general user as the vendor

provides an 'out-of-the-box' informatics solution to capturing, analysing, recording and exporting defined genotypic data into a wide range of analytical software. At the time of writing, the sequence-based methods still require specialised bioinformatics support to collect and interrogate the genotypic data—a big disadvantage for many smaller groups. However, it is a logical development and a significant step forward. Not surprisingly, GbS has already been implemented in barley association mapping studies.

18.4 Marker Ascertainment Issues

Whilst the 'marker constrained' highly multiplex assays such as the OPA and iSELECT technologies from Illumina are tremendously effective and simple to use, they are not ideally suited to all applications. Because their development generally involves mining sequence data extracted from a limited number of individuals, the utility of the SNPs obtained is affected by this discovery protocol. Basically, SNPs are identified in a small panel of individuals selected from a much larger population. As they represent only a small subset of the individuals, only a fraction of total polymorphisms will be discovered. When these SNPs are then scored on a larger sample of individuals, an 'ascertainment bias' is introduced (Nielsen 2000). Because the SNP discovery panel is small, the probability that an SNP will be identified is a function of its frequency in the discovery population. Rare SNPs will go undiscovered more often than common SNPs, and SNPs not present in the discovery population will never be incorporated in the assay platform. When the platform is then used to screen a much broader set of germplasm, this ascertainment bias will compromise measures of relatedness and genetic diversity because statistical measures that rely on allele frequency, such as nucleotide diversity, population genetics parameters and linkage disequilibrium, will be affected (Nielsen 2000; Schlotterer and Harr 2002; Rosenblum and Novembre 2007; Storz and Kelly 2008).

BOPA1, BOPA2 and the 9K iSELECT platforms were developed from SNP data extracted from a limited number of barley accessions (Rostoks et al. 2005, 2006; Close et al. 2009; Comadran et al. 2012), and several large-scale projects have used them effectively to identify marker-trait associations in elite cultivars (AGOUEB, http://www.agoueb.org; Barley CAP, http://barleycap.cfans.umn.edu; ExBarDiv: http://pgrc.ipk-gatersleben.de/barleynet/projects_exbardiv.php) (Waugh et al. 2010) and in diversity panels comprising both elite cultivars and landraces (Pasam et al. 2012). Despite these apparent successes, we should be mindful that the extent and patterns of diversity observed have been affected by ascertainment issues and that results generated in these studies in most cases still need to be validated. This is particularly true when examining diverse genotypes. For example, understanding genetic diversity inherent within accessions that tolerate extreme conditions of temperature and water availability is likely to be particularly important in future breeding efforts that seek to respond to future environmental challenges. It is therefore important that issues such as

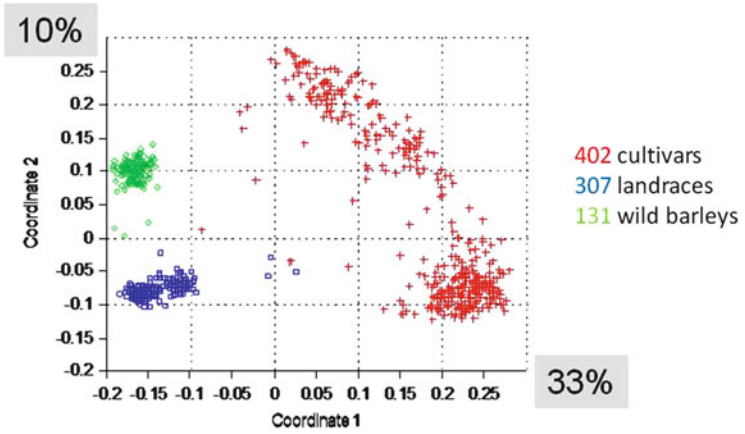

Fig. 18.2 Principle coordinates analysis illustrating the effect of ascertainment bias on estimations of genetic diversity in diverse barley gene pools. The SNPs were ascertained from the cultivated gene pool and were chosen based on high allele frequencies. The wild and landrace barley germplasm 'looks' as if it is much narrower than the cultivated germplasm—which we know from many other studies is completely the wrong way around

ascertainment bias are fully taken into account when using a marker platform derived from one gene pool to investigate another.

One example that highlights this issue and that has been examined in some detail is the use of SNPs sampled from the cultivated gene pool to examine diversity in collections of landrace barleys from Syria and Jordan (Fig. 18.2). Moragues et al. (2010) evaluated the effects of SNP number and selection strategy on estimates of germplasm diversity and population structure in different barley collections. Using the 1,536 BOPA1 SNP data and random or optimised subsets of 384 and 96 SNPs, they compared diversity statistics for 161 landraces from Jordan and Syria with 171 European cultivars that had previously been studied using SSRs (Russell et al. 2003). They observed differences in the patterns of SNP polymorphisms and, somewhat counter-intuitively, a lower estimate of diversity in the landraces, contradicting the SSR results. This bias could be at least partially nullified by selecting an appropriate subset of SNPs.

More recently Russell et al. (2011) described the first application of BOPA1 to assess the evolution of barley in a portion of the Fertile Crescent. Specifically, they were interested in examining diversity across the genome but in particular those regions that have been previously identified as playing a role in domestication. They genotyped geographically matched landrace and wild barleys (448 accessions) from Jordan and Syria. One consequence of ascertainment bias would be to skew the landrace-wild comparison by excluding rarely polymorphic markers in the wild barleys, resulting in an underestimate of their true genetic diversity. However, the experimental data showed higher levels of genetic variation in wild material, and furthermore, the differences were similar to those found in previous work (Russell et al. 2004). Also, if the effect of bias introduced by using SNPs sampled from elite

cultivars was problematic, the expectation would be a reduction of diversity in the wild compared to landraces around the domestication genes (because SNPs in the wild would not have been assayed). But they identified 141 cases where rolling diversity estimates were significantly different between wild and landrace barley genotypes, with diversity higher in wild material for 94 % of the cases, many in regions where domestication genes are known. As ascertainment bias would have pushed this comparison in the other direction, their observations become increasingly significant.

18.5 Accounting for Population Structure

When mapping by association, underlying population structure can be a strong confounding factor that results in a high frequency of false-positive associations. (Rostoks et al. 2006; Mackay and Powell 2007). Considering a hypothetical trait, if this trait was frequently associated with any sub-population, then all corresponding background markers that identify alleles with a similar clustering distribution between populations would also be associated with the trait, regardless of whether they were physically linked to it. Minimising these false-positive effects has been the focus of considerable effort in the statistical genetics community, and a number of approaches have been developed in an attempt to nullify them whilst allowing true associations to be detected.

GWAS analysis that does not account for population substructure (a *naive* approach) is based on the same principles as those applied in biparental QTL mapping populations. Simply, it consists of regressing the phenotype against the alleles at each genetically mapped locus to detect QTLs and is successful because each marker allele in the genetic map has a given probability of being associated with the QTL of interest. The *naive* approach is not generally suitable for use in structured populations for the reasons given above. However, it is suitable for use in populations in which structure has been intentionally minimised. A popular example of this type is a multiparent advanced generation intercross (MAGIC) population (Cavanagh et al. 2008). Another possibility is to use substantially unstructured sub-populations identified by PCO or STRUCTURE analysis of the associated marker data (Waugh et al. 2010), although some would argue that even within these populations, a structure correction should always be applied.

The reality is that barley germplasm sampled across the world is strongly stratified into sub-populations, reflecting growth habit, ear morphology and geographical origin, and is linked to local adaptation and crop end use. As a naive approach is unsuitable in this case, several different statistical approaches that correct and/or account for the effects of population structure within such germplasm have been developed. Indeed, correcting for structure has guided most of the research on GWAS for the last few years (Pritchard et al. 2000; Mackay and Powell 2007). Issues arise when the application of different statistical approaches reveal an inconsistent number and/or identity significant associations or remove known

biological factors that are correlated at some level with population structure. This can result in uncertainty over what QTL to prioritise for further studies or to use as diagnostics in marker-assisted selection (MAS).

Structured association uses genome-wide molecular diversity data to compute statistics that define the genetic structure contained within the germplasm. The derived statistics can then be modelled within a mixed linear model (MLM) framework to account for the multiple levels of relatedness that result from historical stratification and kinship (Yu et al. 2006). Statistical softwares including Genstat (VSN International 2011), R (http://www.R-project.org/) and TASSEL (Bradbury et al. 2007, http://www.maizegenetics.net) can then provide (different) corrections for population structure. A variance covariance matrix containing coefficients of co-ancestry (kinship matrix) can be included in the mixed model to account for genetic relatedness between genotypes. Eigenanalysis (Patterson et al. 2006) uses the scores of the most significant PCA axes from the molecular marker matrix as co-variables in the mixed model, approximating the use of a kinship matrix. In barley Cockram et al. (2010) and Comadran et al. (2011b) found that a mixed linear regression model that accounts for relatedness due to kinship and historical population substructure to perform well. A significance threshold is usually estimated for each analysis using a Bonferroni-corrected p-value of 0.05. Importantly, with the observed increase in marker data volumes, methods that are able to cope with thousands to millions of computationally intensive analyses have emerged that provide a choice of both approximate [e.g. GRAMMAR (Aulchenko et al. 2007), implemented in GenABEL (http://www.genabel.org/packages/GenABEL); P3D (Zhang et al. 2010), implemented in TASSEL (http://www.maizegenetics.net/tassel); EMMAX (Kang et al. 2010) (http://genetics.cs.ucla.edu/emmax/)] and exact methods [e.g. FMM (W. Astle & D. Balding, http://www.genabel.org/MixABEL/FastMixedModel.html); FaST-LMM (Lippert et al. 2011) (http://mscompbio.codeplex.com/); GEMMA (M. Stephens lab, http://stephenslab.uchicago.edu/software.html)] to account for structure effects.

18.6 Data Management and Display

With the size of the datasets generated, both molecular and phenotypic, a key issue for longer-term value of an association mapping population surrounds data management, quality control and data visualisation, particularly if the dataset forms a reference for the wider research community and has been derived from multiple datasets generated by groups from remote locations. Whilst there may be local solutions to this issue, within our programme we have developed and implemented a GERMINATE data warehouse (Lee et al. 2005; http://bioinf.scri.ac.uk/public/?page_id=159) modified to hold high-density phenotypic and genotypic diversity data, Illumina iSELECT and GbS SNP metadata together with the results of our analyses. Working closely with the breeding community has prompted the development of a number of features in GERMINATE that assist data querying,

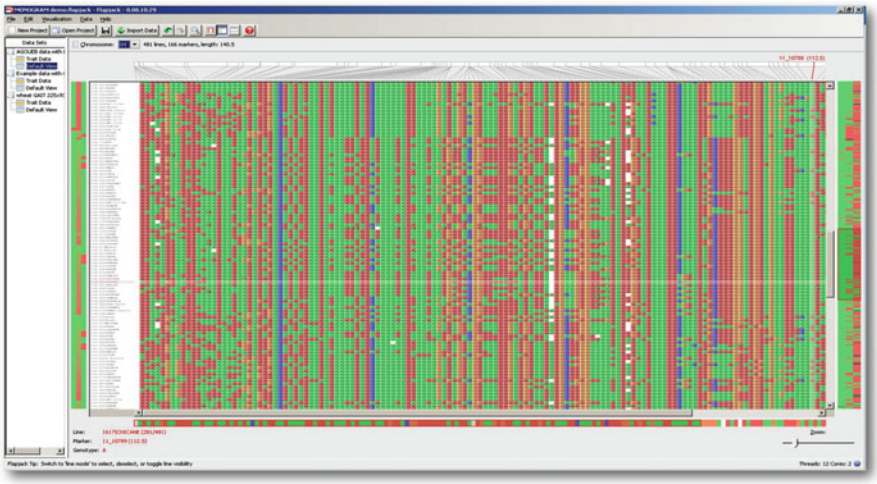

Fig. 18.3 A screenshot of the Flapjack graphical genotyping environment. The marker alleles are colour coded (A, C, G, T, white = missing data) and arranged in genetic marker order along each chromosome (horizontal axis). Individual accessions are shown in the vertical axis. Tracks for visualising trait data are available but not shown. The pattern of SNP alleles along a chromosome can be easily inspected visually (see http://ics.hutton.ac.uk/flapjack/ for further details)

manipulation and visualisation. In particular, interfacing with the Flapjack graphical genotyping environment (Milne et al. 2010) has been of particular significance, with the Flapjack data model (Fig. 18.3) now being widely adopted by other plant breeding and germplasm diversity projects including the 'SeeD' programme at CIMMYT, the Triticeae CAP (T-CAP) project in the United States (http://www.triticeaecap.org/?q=node/2), Gates Foundation-funded GCP Integrated Breeding Platform (http://wiki.cimmyt.org/confluence/display/MBP/Home) and the Gramene Diversity project (http://www.gramene.org/db/diversity/diversity_view). Further developments in these latter projects will enable users to automatically load data and analysis results and provide enhanced tool integration with various genetic analysis platforms. Thus, efforts are underway to more intimately integrate Flapjack with data analysis software such as TASSEL, R, Genstat and genetic simulation tools like QuGene (Podlich and Cooper 1998).

18.7 Phenotypic Analysis

One of the original attractions of association mapping was that it promised to be able to exploit rich phenotypic information that had already been collected either by prior academic studies or of the rigorous trialling and testing procedures that cultivars must go through as part of the official registration process. For example in the United Kingdom, up to 80 morphological-developmental traits are described

and available for use in assessing the distinctiveness, uniformity and stability (DUS) of prospective cultivars and up to 40 (including grain yield, quality and disease resistance) tested for value for cultivation and use (VCU) (http://www.fera.defra.gov.uk/plants/plantVarieties/nationalListing/documents/protocolCereals10.pdf). Work carried out in the AGOUEB population in the United Kingdom and cultivated barley collections at IPK in Germany have reported the use of such data (Cockram et al. 2010; Comadran et al. 2011a; Wang et al. 2012; Matthies et al. 2009, 2012). This may be because it can often be difficult to extract this type of data from archives or because it may be difficult to use as official testing protocols and ways of recording the phenotypic data have been modified over time and accessions may have undergone further selection between the point of testing for DUS/VCU and genotyping. However, where the data are clean, it remains a highly valuable asset that obviates the need for de novo phenotyping. Conducting the necessary quality control prior to analysis is however time consuming and may involve a considerable amount of retesting.

For certain phenotypes, like disease resistance, that are tested on relatively young leaf material using a common 'treatment' (e.g. a pathogen population), morphological-developmental differences between accessions can have limited impact on the collected data. However, the opposite can be true when attempting to collect equivalent data on diverse genotypes that may be confounded by significant developmental and morphological differentiation. For example, wild barley isolates and landraces from around the world have highly diverse heading dates and heights and using data such as 'grain yield' collected in a single environment across such a diverse population may be effectively meaningless. Because of these difficulties we have found it advantageous to 'tune' the accessions in our association mapping population by including only those with broadly similar developmental characteristics. Whilst this necessarily restricts the amount of variation that segregates in the population, we have found that this approach enables rather than restricts genetic dissection of the considerable genetic variation that remains in the population.

18.8 Association Mapping in Barley

Several individual groups and consortia have recently assembled collections of germplasm into association mapping panels and have phenotyped and genotyped them at varying depths with the objective of performing GWAS (e.g. Haseneyer et al. 2010). To date, none are artificially constructed populations such as nested association mapping (NAM; McMullen et al. 2009) or MAGIC (Cavanagh et al. 2008) that are promoted as exploiting the power of both linkage analysis and association mapping approaches and designed to avoid the population structure issues that inflate false-positive associations in natural populations. Such populations are currently under development (http://triticeaecap.org/?q=node/1). Examples of some of the populations already used for GWAS are as follows.

18.8.1 Wild Barley Populations

Steffenson et al. (2007) assembled a Wild Barley Diversity Collection (WDBC) comprising 318 accessions selected on the basis of eco-geographic parameters that included longitude/latitude, elevation, high/low temperature, rainfall and soil type. Most were from the Fertile Crescent, Central Asia, North Africa and the Caucasus region. Single plant selections were repeatedly selfed to near homozygosity and the resulting inbreds genotyped using 558 Diversity Array Technology (DArT®; Jaccoud et al. 2001) and 2,878 BOPA1 and BOPA2 SNPs. GWAS was conducted after correcting for structure, initially for leaf, stem and stripe rust (Steffenson et al. 2007) and latterly for spot blotch (Roy et al. 2010) resistance. 13–15 significant associations of small effect, some corresponding with the location of known resistance genes, were detected for each phenotype. Given the expected extent of LD in the WDBC (Caldwell et al. 2006; Morrell et al. 2005), these results are somewhat surprising and it will be interesting to see if any of the detected associations are subsequently validated. It is tempting to speculate that SNP ascertainment issues, combined with low levels of recombination in the genetic centromeres may have played some role in these findings.

18.8.2 Landraces

A European Union-funded project under the acronym EXBARDIV (http://pgrc.ipk-gatersleben.de/barleynet/projects_exbardiv.php) was founded on the hypothesis that stratified germplasm collections may allow genetic resolution to be manipulated in GWAS by shuttling between cultivated, landrace and wild association mapping populations. The Europe-wide team assembled a collection of 360 elite European barley cultivars (overlapping with the UK AGOUEB Project summarised below), 480 landraces from Jordan and Syria and known as the ICARDA Syrian-Jordanian Landrace Collection (SJLC; Ceccarelli et al. 1987) and two sets of wild barleys, including a subset of 131 individuals from the WBDC summarised above. These lines have been phenotyped for a wide range of characters at multiple sites across Europe and simultaneously genotyped with the barley 9K iSELECT SNP platform. Several manuscripts describing the analysis of the data associated with several of these phenotypes are currently in the pipeline (unpublished). In addition, Casas et al. (2011) surveyed the Spanish Core Collection of barley landraces (Igartua et al. 1998) to identify candidate genes affecting flowering time variation by GWAS. There are, however, few other GWAS studies specifically of barley landraces. Some include landraces as a subset of a wider germplasm collection, e.g. Comadran et al. (2011b), and others have used a limited number of SSR markers, e.g. Jones et al. (2011).

18.8.3 Cultivars

Several populations have been assembled specifically to exploit the potential power of GWAS in cultivated barley material starting with the relatively small population used in the original studies of Kraakman et al. (2004, 2006) and Kraakman (2005). We focus on two of these here. However, whilst we highlight these major efforts, other association mapping populations have been assembled and that have now exploited using the BOPA marker technology. These include MABDE (Comadran et al. 2009), EXBARDIV (see above) and GABI-Genobar (Rode et al. 2012), and results from these are now starting to emerge in the literature.

18.8.3.1 Barley CAP

In order to conduct association mapping (AM) studies of economically important traits in US barley breeding germplasm, a panel of 3,840 US barley breeding lines originating from 10 major breeding programmes was assembled and genotyped with 3,072 SNPs (BOPA1 and BOPA2). Population structure was examined using the programme STRUCTURE (Pritchard et al. 2000) and principle component analysis (PCA), revealing 7–9 sub-populations with some correspondence with the different breeding programmes (Hamblin et al. 2010; Zhou et al. 2012). The major population subdivisions were imposed by inflorescence morphology (two-row versus six-row) growth habit (spring vs. winter) and end use (malt vs. feed). Average LD within sub-populations was found to decay across a range of 20–30 cM in Hamblin et al. (2010) and between 4.0 and 19.8 cM in Zhou and Steffenson (2012) as determined by calculating r^2. The authors estimated that quantitative trait loci (QTL) should be detected in their population with a 50 % probability within a genetic interval of 5 cM and with 95 % probability within 25 cM. These and other studies using subsets of the Barley CAP material (e.g. Cuesta-Marcos et al. 2010; von Zitzewitz et al. 2011; Wang et al. 2012; Massman et al. 2011) and phenotypic data from breeding programmes, were able to detect QTL previously detected in other studies, validating the investment in the association mapping approach. However, none so far have advanced as far as identifying the causal underlying genes. In each of these studies, the authors stress that careful consideration must be given to population diversity, size and experimental design.

18.8.3.2 AGOUEB

The AGOUEB (*pronounced Ag-web*) consortium was established as a public/private partnership in the United Kingdom and was set up to explore the diversity present in European plant breeding programmes using contemporary molecular marker technologies (BOPA1 and BOPA2). Using the same marker platform as

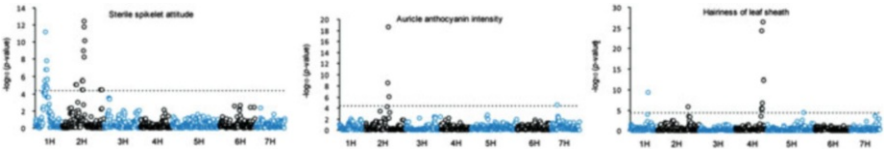

Fig. 18.4 GWAS for three morphological characters—sterile spikelet attitude, auricle anthocyanin intensity and hairiness of the leaf sheath using 1,536 SNPs on a collection of c. 500 mixed barley cultivars (adapted from Cockram et al. 2010). Resolution to single-gene level was achieved for anthocyanin pigmentation where a deletion in *HvbHLH1* was shown to be the causal polymorphism

Barley CAP, Cockram et al. (2010) genotyped a collection of c. 500 cultivars selected from UK registration trials over the past 20 years. As with Barley CAP significant population structure was detected generating high levels of false-positive associations between markers. Significant intrachromosomal LD was observed across the full length of chromosomes (mean distance between significant marker pairs = 40.2 cM, median = 30.7 cM, similar to that observed by Hamblin et al. (2010) in US germplasm). However, after adjustment using a mixed model to take account of population structure, this was reduced to <10 cM (mean = 1.2 cM, median = 0.6 cM), with the proportion of significant inter-chromosomal associations controlled to just 0.1 %. They examined historical phenotypic data for 32 different morphological traits, successfully identifying loci controlling 15 and attributing failure in the other 17 cases to low-quality or variably recorded phenotypic data (e.g. Fig. 18.4). Cockram et al. (2010) also modelled the power to detect 1, 2 and 10 independent loci distributed randomly across the genome, with heritabilities (h^2) of 0.5 and 0.9. Using a mixed model to correct for genetic substructure, simulations based on a trait controlled by one locus predicted that their experimental design had a high probability ($\geq$0.92 for both values of h^2) of detecting significant (q value $\leq$0.1) associations within windows of $\leq$8 cM. However, for a ten-locus trait, they reported that the power to detect one or more loci after correction with the mixed model was low (0.25, $h^2 = 0.5$; 0.58, $h^2 = 0.9$). As with Barley CAP the issues associated with using highly structured populations in AGOUEB were therefore again highlighted as a potential impediment to successful GWAS.

18.9 GWAS to Single Gene Resolution

An advantage of GWAS over the use of biparental populations for trait dissection is that the amount of recombination that has occurred in the population should potentially afford single-gene resolution provided that the gene target does not reside in a genomic region with restricted recombination rate, such as the pericentromeric heterochromatin. Whilst the success of this depends on a large extent on the population assembled, several examples now exist in the literature where this

has indeed turned out to be the case. In *Arabidopsis*, Atwell et al. (2010) provide a number of examples where large-scale phenotyping combined with high-resolution genotyping and GWAS has identified a significant enrichment of a priori candidate genes for a wide range of traits. Thus, Todesco et al. (2010) demonstrated that allelic variation at *ACCELERATED CELL DEATH 6* was responsible for fitness benefits elaborated as resistance to microbial infection and herbivory. However, the same locus also had a marked impact on pleiotropic variation in vegetative growth. In the maize-nested association mapping population, Tian et al. (2011) recently showed that variation in leaf angle and size, parameters that have allowed maize planting density to be increased due to more efficient light capture, is partially controlled by allelic variation at the *LIGULELESS* genes. Similar successes have been achieved in a collection of c. 500 rice landraces (Huang et al. 2010).

In barley there are currently three examples in the literature of the successful use of GWAS to single-gene resolution (Fig. 18.3). In the first, Cockram et al. (2010) clearly demonstrated that this level of resolution was achievable in a germplasm collection comprised of winter and spring, two-rowed and six-rowed elite barley cultivars. By focusing on a robust single-gene phenotype, the presence or absence of anthocyanin pigmentation, they were able to show that variation in the anthocyanin pathway regulatory gene *HvbHLH1* was responsible for the observed phenotype. 'White' alleles contained a diagnostic deletion that resulted in a premature stop codon upstream of the basic helix-loop-helix domain. By assaying for the presence of the deletion in a collection of 'red' and 'white' alleles present in landrace germplasm originating from across Europe, they were able to infer the geographical origin of the white allele and map its subsequent spread throughout Europe.

In the second, Ramsay et al. (2011) were able to identify and prove that *SIX-ROWED SPIKE 5* (*INTERMEDIUM-C*), a gene that affects barley row type, was a functional orthologue of the maize domestication gene *TEOSINTE BRANCHED 1*. They achieved this despite the phenotype being a cause of major population subdivision in the germplasm used in the analysis. Although it is a simple two-state morphological character, GWAS identified four highly significant associations, suggestive of strong epistatic interactions. As would have been predicted, one association peak mapped to the *SIX-ROWED SPIKE 1* (*Vrs1*) locus (Komatsuda et al. 2007), another with *SIX-ROWED SPIKE 5* and the remaining two with separate loci on chromosome 1H. One of these latter loci has subsequently been shown to correspond to the *SIX-ROWED SPIKE 3* locus (our unpublished results). Importantly, Ramsay et al. (2011) were able to validate their candidate gene using a legacy collection of independent spike mutants (Druka et al. 2011) that had previously been attributed to lesions in *SIX-ROWED SPIKE 5* by allelism tests.

Finally Comadran et al. (2012) used a modified analytical approach based on divergent selection between the winter and spring barley gene pools to identify regions of the barley genome where contrasting alleles had been selected in these different lifestyle types. They eventually focussed on one such region which from QTL studies had been called *EARLINESS PER SE 2* and mapped as the major determinant of earliness in a study examining adaptation of barley to droughted

environments. Using available mutant resources they were able to show that the gene responsible for the observed phenotype was the barley orthologue of the *Antirrhinum majus* gene *CENTRORADIALIS,* a paralogue of the *Arabidopsis* flowering repressor *TERMINAL FLOWER 1* (*TFL1*). Within our group we have now used GWAS to identify a number of additional genes and validated them using the same strategy, i.e. with independent barley mutants.

Conclusions

The successes in GWAS-associated identification of gene alleles encoding barley traits described above bode well for the future of this approach, especially since the potential power of the method is continuously increasing. It is not unreasonable to predict that in the next few years, hundreds of thousands of polymorphic sites that are mapped on a reliable physical framework for the barley genome will become available for GWAS in barley. Furthermore, the arrival of GWAS populations with lower substructure, more allelic variation and higher numbers of recombination breakpoints will increase the mapping resolution. In such circumstances single-gene resolution for GWAS will become commonplace.

Future directions of GWAS in barley will to some extent be driven by the falling cost of genotyping associated with next-generation sequencing technologies (NGS). Given the potential to saturate marker coverage of the genome, the discriminatory power of GWAS in barley will be determined by the size of the population studied and the patterns of LD and population structure within the population. The use of large more genetically balanced populations that are specifically developed for GWAS (McMullen et al. 2009; Cavanagh et al. 2008) will undoubtedly play an increasing role though recombination rates in this inbreeding crop will continue to be a limiting factor particularly in certain regions of the genome. In addition to the importance of choice of population, the potential discriminatory power of GWAS will certainly concentrate more attention onto experimental design and the opportunities offered by high-throughput phenotyping. Whilst it is now possible to conduct QTL x environment AM analyses using Genstat (VSN International 2011), current analytical methods are largely single-locus additive models. Future analytical developments will lead to multi-locus models with the potential to detect epistatic interactions, as now in biparental QTL mapping. Finally the discrimination of GWAS in barley down to the gene level will also necessitate the further development of validation strategies and the integration of future population studies with developments in functional genomics and systems analyses in the crop.

To avoid the majority of the potential issues with population substructuring, we have assembled a population of approaching 1,000 two-rowed spring barley varieties that exhibit low population substructure

(continued)

and show similar morpho-developmental characteristics (particularly flowering time). We are currently using this population extensively to investigate a range of simple and more complex traits, and our experience to date suggests that such populations do simplify underlying genetic complexity making it more amenable to statistical interpretation (Waugh et al. 2010). This population is a powerful resource for future genetic analysis in barley, and we welcome collaboration with groups who would like to exploit the power and resolution it affords.

References

Atwell S, Huang YS, Vilhjálmsson BJ, Willems G, Horton M, Li Y, Meng D, Platt A, Tarone AM, Hu TT, Jiang R, Muliyati NW, Zhang X, Amer MA, Baxter I, Brachi B, Chory J, Dean C, Debieu M, de Meaux J, Ecker JR, Faure N, Kniskern JM, Jones JD, Michael T, Nemri A, Roux F, Salt DE, Tang C, Todesco M, Traw MB, Weigel D, Marjoram P, Borevitz JO, Bergelson J, Nordborg M (2010) Genome-wide association study of 107 phenotypes in Arabidopsis thaliana inbred lines. Nature 465:627–631

Aulchenko YS, de Koning D-J, Haley C (2007) Genomewide rapid association using mixed model and regression: a fast and simple method for genomewide pedigree-based quantitative trait loci association analysis. Genetics 177:577–585

Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES (2007) TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633–2635

Büschges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, van Daelen R, van der Lee T, Diergaarde P, Groenendijk J, Töpsch S, Vos P, Salamini F, Schulze-Lefert P (1997) The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell 88:695–705

Caldwell KS, Russell J, Langridge P, Powell W (2006) Extreme population-dependent linkage disequilibrium detected in an inbreeding plant species, Hordeum vulgare. Genetics 172:557–567

Casas AM, Djemel A, Ciudad FJ, Yahiaoui S, Ponce LJ, Contreras-Moreira B, Gracia MP, Lasa JM, Igartua E (2011) HvFT1 (VrnH3) drives latitudinal adaptation in Spanish barleys. Theor Appl Genet 122:1293–1304

Cavanagh C, Morell M, Mackay I, Powell W (2008) From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. Curr Opin Plant Biol 11:215–221

Ceccarelli S, Grando S, Leur JAG (1987) Genetic diversity in barley landraces from Syria and Jordan. Euphytica 36:389–405

Chutimanitsakun Y, Nipper RW, Cuesta-Marcos A, Cistué L, Corey A, Filichkina T, Johnson EA, Hayes PM (2011) Construction and application for QTL analysis of a Restriction Site Associated DNA (RAD) linkage map in barley. BMC Genomics 2011:4

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney RK, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582

Cockram J, White J, Zuluaga D, Smith D, Comadran J, Macaulay M, Luo ZW, Kearsey MJ, Werner P, Harrap D, Tapsell C, Liu H, Hedley PE, Stein N, Schulte D, Steuernagel B, Marshall DF, Thomas WTB, Ramsay L, Mackay I, Balding DJ, Waugh R, O'Sullivan D (2010)

Genome-wide association mapping of morphological traits to candidate gene resolution in the un-sequenced barley genome. Proc Natl Acad Sci U S A 107:21611–21616

Comadran J, Thomas WT, van Eeuwijk FA, Ceccarelli S, Grando S, Stanca AM, Pecchioni N, Akar T, Al-Yassin A, Benbelkacem A, Ouabbou H, Bort J, Romagosa I, Hackett CA, Russell JR (2009) Patterns of genetic diversity and linkage disequilibrium in a highly structured Hordeum vulgare association-mapping population for the Mediterranean basin. Theor Appl Genet 119:175–187

Comadran J, Ramsay L, MacKenzie K, Hayes P, Close TJ, Muehlbauer G, Stein N, Waugh R (2011a) Patterns of polymorphism and linkage disequilibrium in cultivated barley. Theor Appl Genet 122:523–531

Comadran J, Russell JR, Booth A, Pswarayi A, Ceccarelli S, Grando S, Stanca AM, Pecchioni N, Akar T, Al-Yassin A, Benbelkacem A, Ouabbou H, Bort J, van Eeuwijk FA, Thomas WT, Romagosa I (2011b) Mixed model association scans of multi-environmental trial data reveal major loci controlling yield and yield related traits in Hordeum vulgare in Mediterranean environments. Theor Appl Genet 122:1363–1373

Comadran J, Kilian B, Russell J, Ramsay L, Stein N, Ganal M, Shaw P, Bayer M, Thomas WTB, Marshall D, Hedley P, Tondelli A, Pecchioni N, Francia E, Korzun V, Walther A, Waugh R (2012) A homologue of Antirrhinum *CENTRORADIALIS* is a component of the quantitative photoperiod and vernalization independent *EARLINESS PER SE 2* locus in cultivated barley. Nat Genet 44:1388–1392

Cuesta-Marcos A, Szucs P, Close TJ, Filichkin T, Muehlbauer GJ, Smith KP, Hayes PM (2010) Genome-wide SNPs and re-sequencing of growth habit and inflorescence genes in barley: implications for association mapping in germplasm arrays varying in size and structure. BMC Genomics 11:707

Davey JW, Hohenlohe PA, Etter PD, Boone JQ, Catchen JM, Blaxter ML (2011) Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nat Rev Genet 12:499–510

Druka A, Franckowiak J, Lundqvist U, Bonar N, Alexander J, Houston K, Radovic S, Shahinnia F, Vendramin V, Morgante M, Stein N, Waugh R (2011) Genetic dissection of barley morphology and development. Plant Physiol 155:617–627

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell Sharon E (2011) A robust, simple genotyping-by-sequencing (gbs) approach for high diversity species. PLoS One 6:e19379

Fan JB, Oliphant A, Shen R, Kermani BG, Garcia F, Gunderson KL, Hansen M, Steemers F, Butler SL, Deloukas P, Galver L, Hunt S, Mcbride C, Bibikova M, Rubano T, Chen J, Wickham E, Doucet D, Chang W, Campbell D, Zhang B, Kruglyak S, Bentley D, Haas J, Rigault P, Zhou L, Stuelpnagel J, Chee MS (2003) Highly parallel SNP genotyping. Cold Spring Harb Symp Quant Biol LXVIII:69–78

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Wenzel G, Herrmann RG (1990) Construction of an RFLP map of barley. Theor Appl Genet 83:250–256

Graner A, Streng S, Kellermann A, Schiemann A, Bauer E, Waugh R, Pellio B, Ordon F (1998) Molecular mapping and genetic fine-structure of the rym5 locus encoding resistance to different strains of the barley yellow mosaic virus complex. Theor Appl Genet 98:285–290

Hamblin MT, Close TJ, Bhat PR, Chao S, Kling JG, Joseph Abraham K, Blake T, Brooks WS, Cooper B, Griffey CA, Hayes PM, Hole DJ, Horsley RD, Obert DE, Smith KP, Ullrich SE, Muehlbauer GJ, Jannink JL (2010) Population structure and linkage disequilibrium in U.S. barley germplasm: implications for association mapping. Crop Sci 50:556

Haseneyer G, Stracke S, Paul C, Einfeldt C, Broda A, Piepho HP, Graner A, Geiger HH (2010) Population structure and phenotypic variation of a spring barley world collection set up for association studies. Plant Breed 129:271–279

Huang X, Wei X, Sang T, Zhao Q, Feng Q, Zhao Y, Li C, Zhu C, Lu T, Zhang Z, Li M, Fan D, Guo Y, Wang A, Wang L, Deng L, Li W, Lu Y, Weng Q, Liu K, Huang T, Zhou T, Jing Y,

Li W, Lin Z, Buckler ES, Qian Q, Zhang QF, Li J, Han B (2010) Genome-wide association studies of 14 agronomic traits in rice landraces. Nat Genet 42:961–967

Igartua E, Gracia MP, Lasa JM, Medina B, Molina-Cano JL, Montoya JL, Romagosa I (1998) The Spanish barley core collection. Genet Resour Crop Evol 45:475–481

Jaccoud D, Peng K, Feinstein D, Kilian A (2001) Diversity arrays: a solid state technology for sequence information independent genotyping. Nucleic Acids Res 29:E25

Jones H, Civáň P, Cockram J, Leigh FJ, Smith LM, Jones MK, Charles MP, Molina-Cano JL, Powell W, Jones G, Brown TA (2011) Evolutionary history of barley cultivation in Europe revealed by genetic analysis of extant landraces. BMC Evol Biol 11:320

Kang HM, Sul JH, Service SK, Zaitlen NA, Kong SY, Freimer NB, Sabatti C, Eskin E (2010) Variance component model to account for sample structure in genome-wide association studies. Nat Genet 42:348–354

Kim S, Plagnol V, Hu TT, Toomajian C, Clark RM, Ossowski S, Ecker JR, Weigel D, Nordborg M (2007) Recombination and linkage disequilibrium in *Arabidopsis thaliana*. Nat Genet 39:1151–1155

Kleinhofs A, Kilian A, Saghai Marrof MA, Biyashev RM, Hayes P, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705–712

Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Matsumoto T, Yano M (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I–class homeobox gene. Proc Natl Acad Sci U S A 104:1424–1429

Kraakman ATW (2005) Mapping of yield, yield stability, yield adaptability and other traits in barley using linkage disequilibrium mapping and linkage analysis. PhD Dissertation 3772, Wageningen University

Kraakman ATW, Niks RE, Van den Berg PMMM, Stam P, Van Eeuwijk FA (2004) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics 168:435–446

Kraakman ATW, Martinez F, Mussiraliev B, van Eeuwijk FA, Niks RE (2006) Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars. Mol Breed 17:41–58

Lee JM, Davenport GF, Marshall D, Ellis TH, Ambrose MJ, Dicks J, van Hintum TJ, Flavell AJ (2005) GERMINATE: a generic database for integrating genotypic and phenotypic information for plant genetic resource collections. Plant Physiol 139:619–631

Lippert C, Listgarten J, Liu Y, Kadie CM, Davidson RI, Heckerman D (2011) FaST linear mixed models for genome-wide association studies. Nat Methods 8:833–835

Mackay I, Powell W (2007) Methods for linkage disequilibrium mapping in crops. Trends Plant Sci 12:57–63

Malysheva-Otto LV, Ganal MW, Röder MS (2006) Analysis of molecular diversity, population structure and linkage disequilibrium in a worldwide survey of cultivated barley germplasm (*Hordeum vulgare* L). BMC Genet 7:6

Massman J, Cooper B, Horsley R, Neate S, Dill-Macky R, Chao S, Dong Y, Schwarz P, Muehlbauer GJ, Smith KP (2011) Genome-wide association mapping of Fusarium head blight resistance in contemporary barley breeding germplasm. Mol Breed 27:439–454

Matthies IE, Weise S, Roder MS (2009) Association of haplotype diversity in the alpha-amylase gene amy1 with malting quality parameters in barley. Mol Breed 23:139–152

Matthies IE, Sharma S, Weise S, Roder MS (2012) Sequence variation in the barley genes encoding sucrose synthase I and sucrose phosphate synthase II, and its association with variation in grain traits and malting quality. Euphytica 184:73–83

McMullen MD, Kresovich S, Villeda HS, Bradbury P, Li H, Sun Q, Flint-Garcia S, Thornsberry J, Acharya C, Bottoms C, Brown P, Browne C, Eller M, Guill K, Harjes K, Kroon D, Lepak N,

Mitchell SE, Peterson B, Pressoir G, Romero S, Rosas OM, Salvo S, Yates H, Hanson M, Jones E, Smith S, Glaubitz JC, Goodman M, Ware D, Holland JB, Buckler ES (2009) Genetic properties of the maize nested association mapping population. Science 325:737–740

Milne I, Shaw P, Stephen G, Bayer M, Cardle L, Thomas WTB, Flavell AJ, Marshall DF (2010) Flapjack – graphical genotype visualization. Bioinformatics 26:3133–3134. doi:10.1093/bioinformatics/btq580#_blank

Moragues M, Comadran J, Waugh R, Milne I, Flavell AJ, Russell JR (2010) Effects of ascertainment bias and marker number on estimations of barley diversity from high throughput SNP genotype data. Theor Appl Genet 120:1525–1534

Morrell PL, Toleno DM, Lundy KE, Clegg MT (2005) Low levels of linkage disequilibrium in wild barley (Hordeum vulgare ssp. spontaneum) despite high rates of self-fertilization. Proc Natl Acad Sci U S A 102:2442–2447

Nielsen R (2000) Estimation of population parameters and recombination rates from single nucleotide polymorphisms. Genetics 154:931–942

Nilsson NO, Säll T, Bengtsson BO (1993) Chiasma and recombination data in plants: are compatible? Trends Genet 9:344–348

Nordborg M, Borevitz JO, Bergelson J, Berry CC, Chory J, Hagenblad J, Kreitman M, Maloof JN, Noyes T, Oefner PJ, Stahl EA, Weigel D (2002) The extent of linkage disequilibrium in *Arabidopsis thaliana*. Nat Genet 30:190–193

Nordborg M, Hu TT, Ishino Y, Jhaveri J, Toomajian C, Zheng H, Bakker E, Calabrese P, Gladstone J, Goyal R, Jakobsson M, Kim S, Morozov Y, Padhukasahasram B, Plagnol V, Rosenberg NA, Shah C, Wall JD, Wang J, Zhao K, Kalbfleisch T, Schulz V, Kreitman M, Bergelson J (2005) The pattern of polymorphism in *Arabidopsis thaliana*. PLoS Biol 3:e196

Pasam RK, Sharma R, Malosetti M, van Eeuwijk FA, Haseneyer G, Kilian B, Graner A (2012) Genome-wide association studies for agronomical traits in a world-wide spring barley collection. BMC Plant Biol 12:16

Patterson N, Price AL, Reich D (2006) Population structure and eigenanalysis. PLoS Genet 2:e190

Podlich DW, Cooper M (1998) QU-GENE: a platform for quantitative analysis of genetic models. Bioinformatics 14:632–653

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Ramsay L, Comadran J, Druka A, Marshall DF, Thomas WTB, Macaulay M, MacKenzie K, Simpson CG, Fuller J, Bonar N, Hayes PM, Lundqvist U, Franckowiak JD, Close TJ, Muehlbauer G, Waugh R (2011) INTERMEDIUM-C, a modifier of lateral spikelet fertility in barley is an ortholog of the maize domestication gene TEOSINTE BRANCHED 1. Nat Genet 43:169–172

Remington DL, Thornsberry JM, Matsuoka Y, Wilson LM, Whitt SR, Doebley J, Kresovich S, Goodman MM, Buckler ES IV (2001) Structure of linkage disequilibrium and phenotypic associations in the maize genome. Proc Natl Acad Sci U S A 98:11479–11484

Rode J, Ahlemeyer J, Friedt W, Ordon F (2012) Identification of marker-trait associations in the German winter barley breeding gene pool (*Hordeum vulgare* L.). Mol Breed 30:831–843. doi:10.1007/s11032-011-9667-6

Rosenblum EB, Novembre J (2007) Ascertainment bias in spatially structured populations: a case study in the eastern fence lizard. J Hered 98:331–336

Rostoks N, Mudie S, Cardle L, Russell JR, Ramsay L, Booth A, Svensson JT, Wanamaker SI, Walia H, Rodriguez EM, Hedley PE, Liu H, Morris J, Close TJ, Marshall DF, Waugh R (2005) Genome wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. Mol Genet Genomics 274:515–527

Rostoks N, Ramsay L, MacKenzie K, Cardle L, Bhat PR, Roose ML, Svensson JT, Stein N, Varshney RK, Marshall DF, Graner A, Close TJ, Waugh R (2006) Recent history of artificial outcrossing facilitates whole genome association mapping in elite crop varieties. Proc Natl Acad Sci U S A 103:18656–18661

Roy JK, Smith KP, Muehlbauer GJ, Chao S, Close TJ, Steffenson BJ (2010) Association mapping of spot blotch resistance in wild barley. Mol Breed 26:243–256

Russell J, Booth A, Fuller JD, Baum M, Ceccarelli S, Grando S, Powell W (2003) Patterns of polymorphism detected in the chloroplast and nuclear genomes of barley landraces sampled from Syria and Jordan. Theor Appl Genet 107:413–421

Russell J, Booth A, Fuller F, Harrower B, Hedley P, Machray G, Powell W (2004) A comparison of sequence-based polymorphism and haplotype content in transcribed and anonymous regions of the barley genome. Genome 47:389–398

Russell JR, Dawson IK, Flavell AJ, Steffenson B, Weltzien E, Booth A, Ceccarelli S, Grando S, Waugh R (2011) Analysis of more than 1,000 SNPs in geographically-matched samples of landrace and wild barley indicates secondary contact and chromosome-level differences in diversity around domestication genes. New Phytol 191:564–578

Schlotterer C, Harr B (2002) Single nucleotide polymorphisms derived from ancestral populations show no evidence for biased diversity estimates in Drosophila melanogaster. Mol Ecol 11:947–950

Steffenson BJ, Olivera P, Roy JK, Jin Y, Smith KP, Muehlbauer GJ (2007) A walk on the wild side: mining wild wheat and barley collections for rust resistance genes. Aust J Agric Res 2007:532–544

Stein N, Perovic D, Kumlehn J, Pellio B, Stracke S, Streng S, Ordon F, Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive Bymovirus resistance in Hordeum vulgare (L.). Plant J 42:912–922

Storz JF, Kelly JK (2008) Effects of spatially varying selection on nucleotide diversity and linkage disequilibrium: insights from deer mouse globin genes. Genetics 180:367–379

Thomas WTB (2003) Prospects for molecular breeding of barley. Ann Appl Biol 142:1–12

Tian F, Bradbury PJ, Brown PJ, Hung H, Sun Q, Flint-Garcia S, Rocheford TR, McMullen MD, Holland JB, Buckler ES (2011) Genome-wide association study of leaf architecture in the maize nested association mapping population. Nat Genet 43:159–162

Todesco M, Balasubramanian S, Hu TT, Traw MB, Horton M, Epple P, Kuhns C, Sureshkumar S, Schwartz C, Lanz C, Laitinen RAE, Huang Y, Chory J, Lipka V, Borevitz JO, Dangl JL, Bergelson J, Nordborg M, Weigel D (2010) Natural allelic variation underlying a major fitness trade-off in Arabidopsis thaliana. Nature 465:632–636

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. Science 310:1031–1034

von Zitzewitz J, Cuesta-Marcos A, Condon F, Castro AJ, Chao S, Corey A, Filichkin T, Fisk SP, Gutierrez L, Haggard K, Karsai I, Muehlbauer GJ, Smith KP, Veisz O, Hayes PM (2011) The genetics of winterhardiness in barley: perspectives from genome-wide association mapping. Plant Genome 4:76–91

VSN International (2011) GenStat for Windows (14th edn). VSN International, Hemel Hempstead (Web page: GenStat.co.uk)

Wang M, Jiang N, Jia TY, Leach L, Cockram J, Comadran J, Shaw P, Waugh R, Luo ZW (2012) Genome-wide association mapping of agronomic and morphologic traits in highly structured populations of barley cultivars. Theor Appl Genet 124:233–246

Waugh R, Jannink JL, Muehlbauer GJ, Ramsay L (2009) The emergence of whole genome association scans in barley. Curr Opin Plant Biol 12:218–222

Waugh R, Marshall DF, Thomas WTB, Comadran J, Russell JR, Close T, Stein N, Hayes P, Muehlbauer G, Cockram J, O'Sullivan D, Mackay I, Flavell AJ, AGOUEB, BarleyCAP, Ramsay L (2010) Whole-genome association mapping in elite inbred crop varieties. Genome 53:967–972

Wei F, Gobelman-Werner K, Morroll SM, Kurth J, Mao L, Wing R, Leister D, Schulze-Lefert P, Wise RP (1999) The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosome 5S (1HS) of barley. Genetics 153:1929–1948

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3FT*. Proc Natl Acad Sci U S A 103:19581–19586

Yan J, Shah T, Warburton ML, Buckler ES, McMullen MD, Crouch J (2009) Genetic characterization and linkage disequilibrium estimation of a global maize collection using SNP markers. PLoS One 4:e8451

Yu J, Pressoir G, Briggs WH, Bi IV, Yamasaki M, Doebley JF, McMullen MD, Gaut BS, Holland JB, Kresovich S, Buckler ES (2006) A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat Genet 38:203–208

Zhang LY, Marchand S, Tinker NA, Belzile F (2009) Population structure and linkage disequilibrium in barley assessed by DArT markers. Theor Appl Genet 119:43–52

Zhang Z, Eroz E, Lai C-Q, Todhunter HK, Gore MA, Bradbury PJ, Yu J, Arnett DK, Ordovas JM, Buckler ES (2010) Mixed linear model approach adapted for genome-wide association studies. Nat Genet 42:355–360

Zhou H, Muehlbauer G, Steffenson B (2012) Population structure and linkage disequilibrium in elite barley breeding germplasm from the United States. J Zhejiang Univ Sci B 13:438–451

Chapter 19
Genomic Selection in Barley Breeding

Karl J. Schmid and Patrick Thorwarth

19.1 Introduction

A central goal of plant breeding is the improvement of plant yield and of traits that facilitate plant production in modern agricultural systems. Traits like disease resistance are often controlled by single genes, but most traits are quantitative and influenced by multiple genes, or quantitative trait loci (QTLs). Plant breeding was mainly based on phenotypic selection, and the availability and decreasing costs of genetic markers enabled marker-assisted selection (MAS) of predominately qualitative traits that are controlled by a small number of genes with large effects (Kandemir et al. 2000). Long before it was technically feasible, Lande and Thompson (1990) recognised the potential of dense genotyping to overcome some limitations of QTL-based marker-assisted selection, for the improvement of breeding populations. Subsequently, this insight led to a new breeding method, called Genomic Selection (GS), which uses genome-wide sets of genetic markers to predict the breeding value of individuals for selection in breeding programmes. With current genome analysis tools such as next generation sequencing (NGS) in hand, the sequencing of whole genomes and large-scale genotyping of many individuals became possible (Metzker 2010; Elshire and Glaubitz 2011). Since 2010, large numbers of individuals of model plants like *Arabidopsis thaliana* and of major crops like rice and maize have been sequenced. In each species, millions of single nucleotide polymorphisms (SNPs), copy number variants (CNVs) and other structural variants were discovered that are available as genetic markers. The key advantages of GS as a new breeding method are a reduced dependency on costly phenotypic selection, the increase of the genetic gain per selection cycle and shorter

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breeding cycles. The purpose of this review is to present Genomic Selection as a breeding method and to evaluate its prospects for barley breeding.

19.2 A Brief Outline of Genomic Selection

Genome-based selection was described by Meuwissen et al. (2001) as a selection strategy that offered a promising tool to exploit genes with small effects on phenotypic variation. Genomic Selection, as this method was called, was initially developed for animal breeding (Hayes et al. 2009a), where it quickly became the method of choice, particularly in cattle breeding because of enormous savings of cost and time. The success of this breeding method led to the evaluation of GS for different crop plants and breeding systems like hybrid breeding or line breeding (Bernardo and Yu 2007; Heffner et al. 2009; Resende et al. 2012b). GS enables the prediction of breeding values by the estimation of marker effects of the breeding material, based on the genotypic and phenotypic information of a calibration (or training) population (Jannink et al. 2010) used to train a prediction model (Fig. 19.1). Based on a model and its parameters, the genomic estimated breeding value (GEBV) of individuals in the breeding population is calculated only from the genotypic data in subsequent selection cycles. This step is also called genomic prediction (GP). The Genomic Selection of breeding individuals can be carried out soon after germination because only DNA needs to be harvested and genotyped.

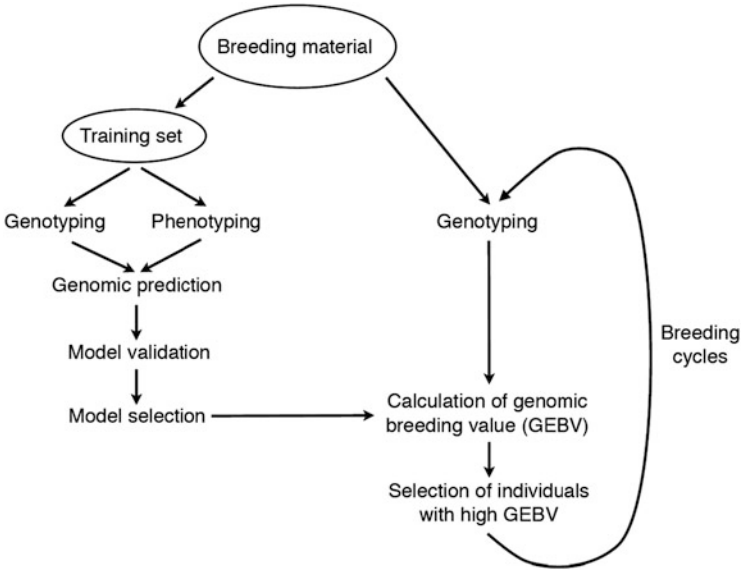

Fig. 19.1 A schematic outline of the genomic selection process and its integration into a breeding programme

Therefore, costs of breeding programmes are reduced, and the selection gain per time unit is higher than in classical phenotype-based breeding programmes (König et al. 2009).

19.3 The Role of Linkage Disequilibrium in Genomic Selection

GS does not require the prior mapping of QTLs that affect the desired trait because it assumes that given a sufficient density of markers, all QTLs affecting the trait of interest are in linkage disequilibrium (LD) with at least one marker used for the GEBV calculation. In other words, each marker tags a section of the genome (or chromosome) that may contain a QTL. As a consequence, the marker density used in GS must be high enough to fulfil this assumption. To estimate the GEBV of individuals, the effects of all marker-tagged genome segments are summed up with the following basic equation of Meuwissen et al. (2001):

$$\text{GEBV} = \sum_{i}^{z} \boldsymbol{X}_i \hat{\boldsymbol{g}}_i \tag{19.1}$$

where z is the number of chromosome segments across the genome (e.g. the number of markers used), $\boldsymbol{X}_i$ a design matrix which is used to assign individuals from the test population to the marker effects of segment i and $\hat{\boldsymbol{g}}$ the vector of effects of the different markers in segment i. The equation has the important property that effects of all markers are estimated at the same time, which is in contrast to QTL mapping, where marker effects are estimated independently. The dependency of the GEBV estimation on a high marker density also requires to have some a priori information about the genome size of the species, the average level of LD, allele frequencies and the population structure to select the optimal numbers of markers and individuals for the calibration population. Another feature of Eq. (19.1) is that GEBV estimation is possible with single markers, but also with predefined haplotypes (i.e. groups of markers in a genomic region), or identity-by-descend (IBD) segments (i.e. genomic regions that have a common ancestry among individuals). Different models exist for each of these options. GS is essentially a 'black box' method, and in contrast to MAS, it is not required to know which marker is associated with a QTL, which therefore remains anonymous. However, the efficiency of GS depends on the extent of genome-wide LD, marker density, the heritability of the selected trait and the size of the calibration population.

19.4 Quantitative Genetic Models Used in Genomic Selection

The success of the GS method depends on the quality of the statistical model used for calculating the GEBV of individuals from phenotypic and genotypic data of the calibration population. Genomic prediction models try to capture as much genetic variation as possible, unlike MAS that utilises only a small proportion of the total genetic variation. Numerous methods for genomic prediction were developed that differ by their statistical models (e.g. linear model regression, Bayesian approaches), in their assumptions about the genetic architecture of a trait, and how the relationship of individuals in a population is accounted for (Daetwyler et al. 2008, 2010; Hayes et al. 2010; Clark et al. 2011). In early approaches to GS, marker effects were estimated with multiple linear regression models, which allow the selection of a subset of markers with a significant effect on the phenotype that can be used in the selection step (Lorenzana and Bernardo 2009). The Best Linear Unbiased Prediction (BLUP) technique models the genetic relationship of individuals based on pedigree information with a variance-covariance matrix (Henderson 1973; Piepho et al. 2007). For this reason, the kinship and population structure information is useful for calculating GEBV and can either be derived directly from markers (e.g. GBLUP) or from pedigrees (e.g. PBLUP). A key difference among statistical models for genomic prediction is the assumption of how the marker effects of the different chromosome segments (i.e. markers) are distributed. Since little a priori information exists about this method, all statistical models are heuristic to some degree, and their prediction ability should be evaluated in comparative validation studies. For illustration, the Genomic Best Linear Unbiased Prediction (GBLUP) is outlined in greater detail. It utilises all marker information to calculate the realised relationship matrix to derive the true genetic relationship of the individuals within a population (Meuwissen et al. 2001; Hayes et al. 2009b) and is written as

$$\boldsymbol{y} = \boldsymbol{X}\beta + Z\boldsymbol{u} + e \tag{19.2}$$

where $\boldsymbol{y}$ is a vector of phenotypic observations, β is the fixed effects vector, $\boldsymbol{X}$ is a design matrix relating the observations to the fixed effects β, $\boldsymbol{u}$ is the vector of the random effects with $\boldsymbol{u} \sim N(0, \boldsymbol{U}\sigma_u^2)$. The realised relationship matrix $\boldsymbol{U}$ is calculated, in the case of completely homozygous individuals, as the simple matching coefficient (S_{SM}) (Reif et al. 2005). The value of a single entry of the matrix is derived as the number of shared alleles across loci between two lines i and j. A closely related method is Ridge Regression BLUP (RR-BLUP), which performs a regression of the phenotypic observations on the marker information by applying a so-called shrinkage factor to reduce over-parametrisation. The RR-BLUP method assumes that every marker has an effect and that the variance of each marker effect is equal (Whittaker et al. 2000; Piepho 2009; Zhang et al. 2010). To overcome the unrealistic assumption of marker effects with equal variances, Bayesian methods were

developed to allow marker-specific shrinkage and the use of a priori information about the distribution of the marker effects. Common methods are BayesA, BayesB (Meuwissen et al. 2001) and the Bayesian Least Absolute Shrinkage and Selection Operator (Bayesian LASSO or BL; de los Campos et al. 2009). The reader is referred to the original publications for further details on these methods.

19.4.1 Cross-Validation: How Good Are Models for Genomic Prediction?

Models for genomic prediction are evaluated and compared in a cross-validation scheme. The key quantity is prediction ability, which is the correlation between observed phenotypic and predicted genotypic values $r(\boldsymbol{y}_{\mathrm{TS}}, \hat{\boldsymbol{g}}_{\mathrm{TS}})$ in the test set (TS). Cross-validation is the repeated calculation of prediction abilities for different test sets extracted from the total set of phenotyped and genotyped individuals using a certain rule (Kohavi 1995). In a typical cross-validation scheme, a data set of corresponding phenotypic and genotypic information is divided into k subsets. From these k subsets, one test set is randomly chosen, and the genotypic values are predicted using the estimated effects of the $k-1$ remaining subsets, which are called estimation set (Albrecht et al. 2011). Various parameters of the GS method such as the (relative) size of the test set or the number of markers for an optimal prediction can be evaluated by varying the quantity of these variables and respectively calculating the prediction ability.

19.4.2 Software for Genomic Prediction

Most methods for genomic prediction were implemented as publicly available software tools. For example, the bayesian linear regression (BLR) (Pérez et al. 2010) and the synbreed packages (Wimmer et al. 2012) were developed for the R statistical environment. The latter package provides access to several current methods for genomic prediction as well as for data processing before prediction and the visualisation of analysis results.

19.5 Genomic Selection in Plants

GS was initially developed for outcrossing species and was mainly applied in cattle breeding programmes where marker effects are assumed to remain constant in different cycles of crossing and selection. In the plant breeding context, GS was first evaluated in simulation studies, which showed that the response to selection in maize can be up to 43 % higher per time unit and more cost-efficient than marker-

assisted recurrent selection (Bernardo and Yu 2007). Similar results were obtained for self-fertilising crops (Piyasatian et al. 2007) and trees (Wong and Bernardo 2008). First empirical studies with *Arabidopsis thaliana* (Lorenzana and Bernardo 2009), barley (Zhong et al. 2009), maize (Crossa et al. 2010) and wheat (de los Campos et al. 2009; Crossa et al. 2010; Heffner et al. 2011) populations demonstrated the superiority of GS over phenotypic or marker-assisted selection. Recent studies evaluated the practical applicability of GS in crop plants (Albrecht et al. 2011; Riedelsheimer et al. 2012; Technow et al. 2012; Zhao et al. 2012) and tree breeding (Resende et al. 2012a, b; Kumar et al. 2012). The predictive ability of all published statistical models for GS was compared with publicly available data from different plant species (*Arabidopsis thaliana*, wheat, barley and maize; Heslot et al. 2012). The key results of the survey can be summarised as follows: (1) The prediction ability between phenotypic values and GEBVs was remarkably similar across data sets and statistical models and ranged from 0.22 to 0.99. Bayesian models did not perform consistently better than BLUP-based models, which are computationally faster. (2) The correlation of the prediction of marker effects was very high between some models (e.g. 0.88 between RR-BLUP and Bayesian LASSO). (3) The outcome of genomic prediction was strongly affected by the presence of a population structure in the data. (4) The combination of different models into a single prediction does not significantly improve prediction ability.

19.6 Genomic Selection in Self-Fertilising Crops

Self-fertilising crops have the advantage that fewer markers are needed to perform prediction of genotypic values in comparison to outcrossing species because of a higher LD (Piyasatian et al. 2007). On the other hand, crosses are more laborious and expensive than in outcrossing crops. Therefore, the most promising prospect of genomic prediction in self-fertilising plant breeding strategies is the deduction of line per se performance with reduced progeny testing. Another application of GS is the production of doubled haploids (DH) to reduce the length of the breeding cycle in self-fertilising species or in general for inbred line production. In DH production, GS could be easily applied to select the best haploid plants before the chromosome-doubling step to reduce the number of individuals for colchicine treatment and subsequent field trials. Bernardo (2010) simulated a barley breeding programme using GS to reduce the number of necessary crosses while maximising the gain of selection per time unit using the scheme shown in Fig. 19.2. The simulations started from recombinant inbred lines (RILs) derived from a cross of two inbred parents. In the breeding scheme, the RIL population is genotyped and evaluated in multi-location field trials in cycle 0, and the phenotypically best RIL lines are selected and intercrossed. The resulting F1 from cycle 0 are selfed, the F2 are genotyped and the best individuals are selected based on the genetic model and intermated again. The resulting F1 from this cross are selfed again, and the F2 selected and intermated to

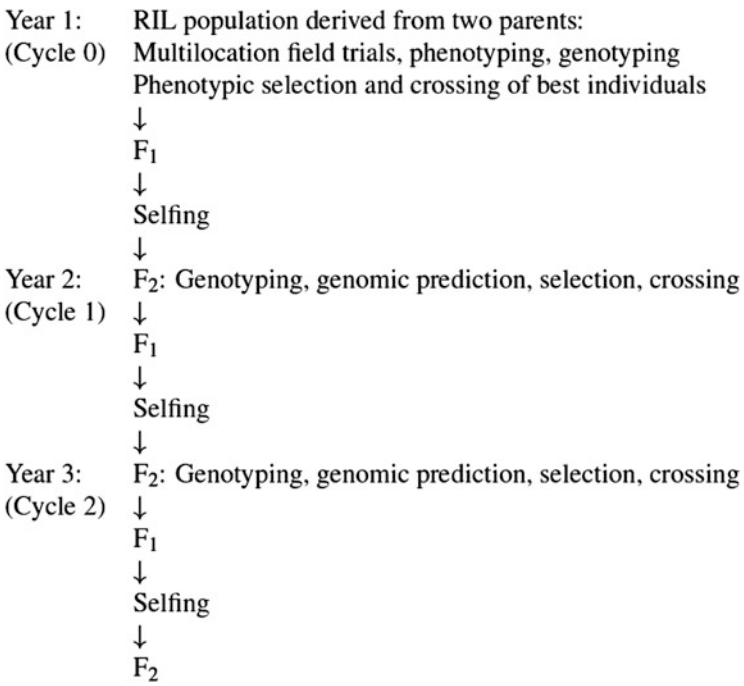

Fig. 19.2 Schematic outline of the breeding scheme used in the simulation by Bernardo (2010)

produce the F3 in the third cycle. The selection, intercrossing and selfing step is repeated once more. By using winter nurseries and greenhouses, 2–3 generations per year can be achieved and three cycles are completed after 3 years. This 'select-recombine-self' scheme was compared against a comparable breeding programme for an outcrossing crop with a 'select-recombine' scheme. The simulations revealed that breeding progress in a selfing species is 81–87 % of the progress achieved with GS in an outcrossing species in the same period of time due to the extended breeding cycle length from the required self-fertilisations. On the other hand, the inclusion of a crossing step in each cycle increased the response to selection severalfold compared to a breeding scheme in which RILs were self-fertilised, and the best lines were selected in each generation based on the markers. The latter scheme does not include recombination among superior offspring and essentially represents the selection of superior RIL lines from cycle 0 over several generations. The simulations also confirmed that small sets of 128 or 256 markers are sufficient for the size of the barley linkage map (1,069 cM) to obtain a significant response to selection even for simulated traits with a heritability as low as 20 %. In these sets, each marker tags on average segments of 8.4 cM and 4.2 cM, respectively, which reflects the high level of LD in the barley genome.

19.7 An Example of Genomic Prediction with German Barley Varieties

As outlined above, the prediction ability of different models for genomic prediction is remarkably similar for different traits and populations. But there are also important differences, and it is instructive to examine different models and model parameters for each breeding population. The barley genome is now covered with a high density of markers, and one can test whether higher marker densities will improve the prediction ability. A relationship matrix based on thousands of SNPs may provide a better estimation of breeding values if it depends mainly on the relationship among individuals. If, on the other hand, a prediction model is more influenced by the extent of linkage between marker and QTL, no improvement above a certain marker density is expected because all QTLs segregating in the population are in significant linkage with at least one marker. To demonstrate the effect of models and marker types on prediction ability, we analysed data from 109 German barley varieties released between 1959 and 2003 (Rode et al. 2012). Ninety varieties were genotyped for 6,808 biallelic SNPs, and we applied genomic prediction for thousand kernel weight (TKW), which is a normally distributed trait with a mean of 47.12 g (SD: 5.40 g) and a heritability of $h^2 = 0.99$ (Thorwarth 2012). The prediction ability with GBLUP and RR-BLUP was 0.78, and 0.76 with Bayesian LASSO. The predicting ability increases with marker number, and BL performed slightly better at low marker numbers than GBLUP, consistent with earlier studies (Meuwissen 2009). On the other hand, a small number of markers is sufficient for GS in barley because the prediction ability of GBLUP was reduced by only 13 % if 95 % (6,467) of the markers were randomly removed (Fig. 19.3a). A similar reduction was observed with Bayesian LASSO. The same behaviour could be observed with other barley populations (e.g. Jannink et al. 2012). If the presence of LD between marker and QTL is the main factor influencing prediction ability, a strong decay of the prediction ability with decreasing marker density would be expected. Since this is not the case, prediction ability may be influenced by a fairly large number of markers with a low LD to causative QTLs, and in addition by a kinship structure present in the sample (Ober et al. 2012). In cultivated barley populations, a high degree of genome-wide LD is usually observed (Caldwell et al. 2006; Zhong et al. 2009), and the small number of markers that are sufficient to obtain high prediction abilities in this example and in other studies (Jannink et al. 2012) likely is caused by a small effective population size due to selective breeding and self-pollination, and additionally a strong population structure in the sample. Furthermore, the number of individuals required for a high prediction ability can also be reduced significantly if the number of markers is kept constant (Fig. 19.3b). For example, the prediction ability increased by only 4.4 % with GBLUP if the number of individuals was increased from 45 to 72, whereas with Bayesian LASSO the prediction ability increased by 8 %. The same trend was also seen in other studies (Asoro et al. 2011; Meuwissen 2009; Jannink et al. 2012). The inclusion of many individuals in genomic prediction, based on cross-validation,

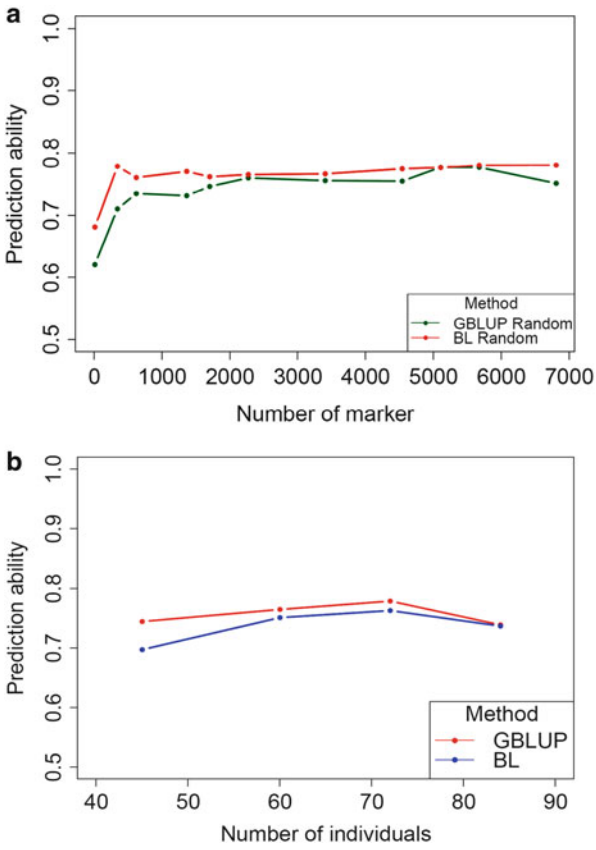

Fig. 19.3 Effect of the numbers of markers (**a**) and individuals (**b**) on the prediction ability with GBLUP in a set of German barley varieties

produces a saturation curve with diminishing returns for larger numbers of individuals, and even a decrease of prediction ability with very large numbers (Jannink et al. 2012). An estimation of the population size required for prediction accuracies of 0.9 can be achieved with the formula $10 \times N_e \times L$ ($10 \times$ effective population size $\times$ genome length in Morgans) (Meuwissen 2009). Since this estimate was obtained from simulations of outcrossing species, Bernardo (2010) suggested that the population size in self-fertilising plants should be doubled in comparison to outcrossing species to achieve comparable accuracies. In summary, several factors influence the quality of genomic prediction: the heritability and the genetic architecture of the trait, the amount of LD, marker density and the sample size of the calibration population (Hayes et al. 2010; Daetwyler et al. 2010; Ober et al. 2012). The genetic variance in the population is captured by using markers in LD with a QTL and by modelling the genetic relationship of the individuals in the population (Jannink et al. 2010). One factor that affects the amount of LD is the number of recombination events (Hamblin et al. 2011), which depends on the effective population size. Since the latter also determines the level of genetic diversity, the extent of genetic diversity in a population also needs to be considered in GS

(Jannink et al. 2010). For example, the offspring from a biparental cross genotyped with a small number of markers (69 simple sequence repeats (SSRs)) gave high prediction accuracies (Lorenzana and Bernardo 2009), but having a larger number of unrelated individuals in a population may require larger calibration populations and more markers. Two additional effects on prediction ability are the method of imputation for missing genotypes and the cross-validation scheme. If the marker density (and therefore LD) is too low, the imputation may be incorrect. The cross-validation scheme could also be a reason for variation in the prediction abilities due to an uneven sampling. Sampling approaches that randomly sample the whole population could be used, or sampling approaches where a sampling based on the genetic relationship among individuals can be used to assess the strength of such an effect (Albrecht et al. 2011; Heslot et al. 2012).

Conclusion

GS in barley breeding is only at the beginning, but the currently available studies of empirical data and simulation studies indicate the great potential of this new breeding method. Since it capitalises on the rapid development in genomics technology as well as in statistical analysis, a fast progress can be expected. Key questions that need to be addressed to improve GS include (1) the size of appropriate training populations, (2) the value of GS to integrate exotic germplasm, (3) the stability of GS predictions over years and environments and (4) the extent and frequency of phenotyping for model building and improvement relative to genotyping. Currently, different traits are considered independently in GS, but in real breeding programmes, selected traits need to be combined into a complex phenotype by creating a selection index for different traits predicted independently with GS or by developing statistical models that allow the simultaneous prediction for several traits. Furthermore, the integration of GS with other breeding methods like MAS needs to be considered if the resulting lines should harbour 'must-have alleles' such as certain disease resistance alleles. The genome-wide selection of SNP alleles may lead to a rapid loss of genetic variation in GS over time, which should be monitored in the course of GS programmes. Finally, the functional annotation of genes in barley genome and the identification of genetic networks in barley may lead to better statistical models that account for additive, dominance, epistatic and other genetic effects in GS.

References

Albrecht T, Wimmer V, Auinger HJ, Erbe M, Knaak C, Ouzunova M, Simianer H, Schön CC (2011) Genome-based prediction of testcross values in maize. Theor Appl Genet 123:339–350

Asoro FG, Newell MA, Beavis WD, Scott MP, Jannink JL (2011) Accuracy and training population design for genomic selection on quantitative traits in elite North American oats. Plant Genome J 4:132

Bernardo R (2010) Genomewide selection with minimal crossing in self-pollinated crops. Crop Sci 50:624
Bernardo R, Yu J (2007) Prospects for genomewide selection for quantitative traits in maize. Crop Sci 47:1082
Caldwell KS, Russell J, Langridge P, Powell W (2006) Extreme population-dependent linkage disequilibrium detected in an inbreeding plant species, Hordeum vulgare. Genetics 172:557–567
Clark SA, Hickey JM, van der Werf JHJ (2011) Different models of genetic variation and their effect on genomic evaluation. Genet Sel Evol 43:18
Crossa J, Campos GDL, Pérez P, Gianola D, Burgueño J, Araus JL, Makumbi D, Singh RP, Dreisigacker S, Yan J, Arief V, Banziger M, Braun HJ (2010) Prediction of genetic values of quantitative traits in plant breeding using pedigree and molecular markers. Genetics 186:713–724
Daetwyler HD, Villanueva B, Woolliams J (2008) Accuracy of predicting the genetic risk of disease using a genome-wide approach. PLoS One 3:e3395
Daetwyler HD, Pong-Wong R, Villanueva B, Woolliams J (2010) The impact of genetic architecture on genome-wide evaluation methods. Genetics 185:1021–1031
de los Campos G, Naya H, Gianola D, Crossa J, Legarra A, Manfredi E, Weigel K, Cotes JM (2009) Predicting quantitative traits with regression models for dense molecular markers and pedigree. Genetics 182:375–385
Elshire R, Glaubitz J (2011) A robust, simple, genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 6:e19379
Hamblin MT, Buckler ES, Jannink JL (2011) Population genetics of genomics-based crop improvement methods. Trends Genet 27:98–106
Hayes BJ, Bowman PJ, Chamberlain AJ, Goddard ME (2009a) Genomic selection in dairy cattle: progress and challenges. J Dairy Sci 92:433–443
Hayes BJ, Visscher PM, Goddard ME (2009b) Increased accuracy of artificial selection by using the realized relationship matrix. Genet Res 91:47–60
Hayes BJ, Pryce J, Chamberlain AJ, Bowman PJ, Goddard ME (2010) Genetic architecture of complex traits and accuracy of genomic prediction: coat colour, milk-fat percentage, and type in Holstein cattle as contrasting model traits. PLoS Genet 6:e1001139
Heffner EL, Sorrells ME, Jannink JL (2009) Genomic selection for crop improvement. Crop Sci 49:1
Heffner E, Jannink J, Sorrells M (2011) Genomic selection accuracy using multifamily prediction models in a wheat breeding program. Plant Genome 4:65–75
Henderson C (1973) Sire evaluation and genetic trends. J Anim Sci 1973:10–41
Heslot N, Yang HP, Sorrells ME, Jannink JL (2012) Genomic selection in plant breeding: a comparison of models. Crop Sci 52:146
Jannink JL, Lorenz AJ, Iwata H (2010) Genomic selection in plant breeding: from theory to practice. Brief Funct Genet 9:166–177
Jannink J, Smith K, Lorenz A (2012) Potential and optimization of genomic selection for Fusarium head blight resistance in six-row barley. Crop Sci 52:1609–1621
Kandemir N, Jones BL, Wesenberg DM, Ullrich SE, Kleinhofs A (2000) Marker-assisted analysis of three grain yield QTL in barley (*Hordeum vulgare* L.) using near isogenic lines. Mol Breed 3:157–167
Kohavi R (1995) A study of cross-validation and bootstrap for accuracy estimation and model selection. Int Joint Conf Artif Intell 14:1137–1145
König S, Simianer H, Willam A (2009) Economic evaluation of genomic breeding programs. J Dairy Sci 92:382–391
Kumar S, Chagné D, Bink MCAM, Volz RK, Whitworth C, Carlisle C (2012) Genomic selection for fruit quality traits in apple (Malus x domestica Borkh.). PLoS One 7:e36674
Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743–756

Lorenzana RE, Bernardo R (2009) Accuracy of genotypic value predictions for marker-based selection in biparental plant populations. Theor Appl Genet 120:151–161
Metzker ML (2010) Sequencing technologies – the next generation. Nat Rev Genet 11:31–46
Meuwissen THE (2009) Accuracy of breeding values of 'unrelated' individuals predicted by dense SNP genotyping. Genet Sel Evol 41:35
Meuwissen TH, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829
Ober U, Ayroles J, Stone E, Richards S, Zhu D (2012) Using whole-genome sequence data to predict quantitative trait phenotypes in Drosophila melanogaster. PLoS Genet 8:e1002685
Pérez P, de Los Campos G, Crossa J, Gianola D (2010) Genomic-enabled prediction based on molecular markers and pedigree using the Bayesian linear regression package in R. Plant Genome 3:106
Piepho HP (2009) Ridge regression and extensions for genomewide selection in maize. Crop Sci 49:1165
Piepho HP, Möhring J, Melchinger AE, Büchse A (2007) BLUP for phenotypic selection in plant breeding and variety testing. Euphytica 161:209–228
Piyasatian N, Fernando RL, Dekkers JCM (2007) Genomic selection for marker-assisted improvement in line crosses. Theor Appl Genet 115:665–674
Reif J, Melchinger A, Frisch M (2005) Genetical and mathematical properties of similarity and dissimilarity coefficients applied in plant breeding and seed bank management. Crop Sci 45:1–7
Resende MDV, Resende MFR, Sansaloni CP, Petroli CD, Missiaggia AA, Aguiar AM, Abad JM, Takahashi EK, Rosado AM, Faria DA, Pappas GJ, Kilian A, Grattapaglia D (2012a) Genomic selection for growth and wood quality in Eucalyptus: capturing the missing heritability and accelerating breeding for complex traits in forest trees. New Phytol 194:116–128
Resende MFR, Muñoz P, Resende MDV, Garrick DJ, Fernando RL, Davis JM, Jokela EJ, Martin TA, Peter GF, Kirst M (2012b) Accuracy of genomic selection methods in a standard dataset of loblolly pine (Pinus taeda L.). Genetics 190:1503–1510
Riedelsheimer C, Czedik-Eysenberg A, Grieder C, Lisec J, Technow F, Sulpice R, Altmann T, Stitt M, Willmitzer L, Melchinger AE (2012) Genomic and metabolic prediction of complex heterotic traits in hybrid maize. Nat Genet 44:217–220
Rode J, Ahlemeyer J, Friedt W, Ordon F (2012) Identification of marker-trait associations in the German winter barley breeding gene pool (Hordeum vulgare L.). Mol Breed 30:831–843
Technow F, Riedelsheimer C, Schrag T, Melchinger A (2012) Genomic prediction of hybrid performance in maize with models incorporating dominance and population specific marker effects. Theor Appl Genet 125(6):1181–1194
Thorwarth P (2012) Genomic prediction in self-fertilizing plants. Master's thesis, University of Hohenheim
Whittaker JC, Thompson R, Denham MC (2000) Marker-assisted selection using ridge regression. Genet Res 75:249–252
Wimmer V, Albrecht T, Auinger HJ, Schön CC (2012) Synbreed: a framework for the analysis of genomic prediction data using R. Bioinformatics 28:2086–2087
Wong CK, Bernardo R (2008) Genomewide selection in oil palm: increasing selection gain per unit time and cost with small populations. Theor Appl Genet 116:815–824
Zhang Z, Liu J, Ding X, Bijma P, de Koning DJ, Zhang Q (2010) Best linear unbiased prediction of genomic breeding values using a trait-specific marker-derived relationship matrix. PLoS One 5:1–8
Zhao Y, Gowda M, Longin F, Würschum T, Ranc N, Reif J (2012) Impact of selective genotyping in the training population on accuracy and bias of genomic selection. Theor Appl Genet 125 (4):707–713
Zhong S, Dekkers JCM, Fernando RL, Jannink JL (2009) Factors affecting accuracy from genomic selection in populations derived from multiple inbred lines: a barley case study. Genetics 182:355–364

Chapter 20
Haploid Technology

Jochen Kumlehn

20.1 Introduction

Haploid technology has proven its worth as a rapid means of effecting crop improvement. Since each doubled haploid plant originates from a different gametophytic cell, a population of doubled haploid plants represents a collection of meiotically recombined but genetically fixed individuals. Doubled haploid populations provide both an ideal source of genetic variation from which to select superior genotypes and a convenient resource for genetic mapping. The rapid attainment of homozygosity is also useful in the context of fixing the outcomes of interspecific recombination, induced mutagenesis and transgenesis. Barley (*Hordeum vulgare*) is amenable to at least two routes of haploid plant production, i.e. either via in vitro culture of immature pollen or via uniparental genome elimination following interspecific hybridisation. The use of haploid technology in the cereals was pioneered in barley and has enjoyed widespread use in both applied and basic barley research.

20.2 The Generation of Plants from Haploid Cells

The capacity of a haploid founder cell to successfully differentiate into a haploid plant forms the basis of haploid technology. Doubled haploids are not only homozygous at all loci, but in addition, since the founder cells are the product of meiosis, the doubled haploid derivatives will differ from one another genotypically (Fig. 20.1). In the flowering plants, female gametophytes (i.e. embryo sacs) develop

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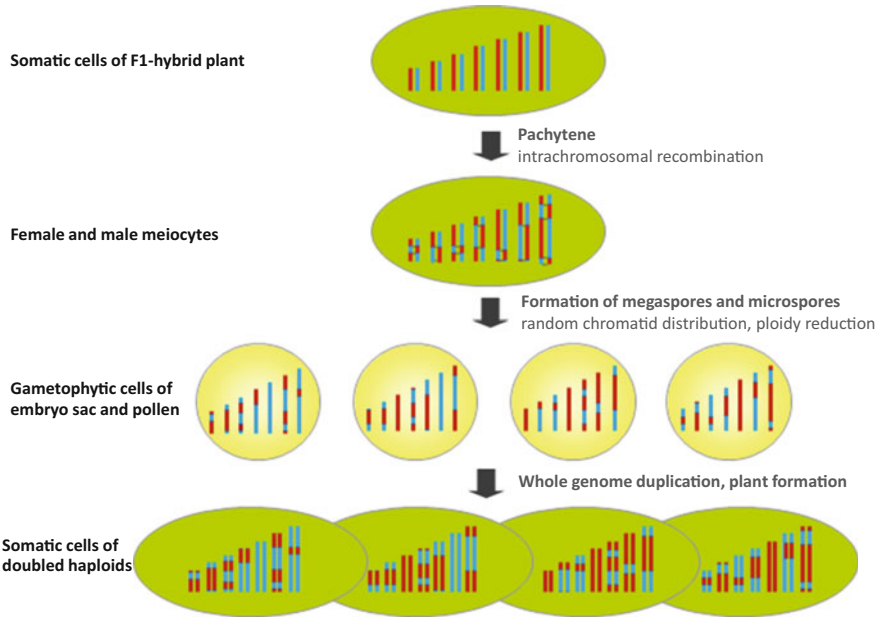

Fig. 20.1 Genetic recombination and fixation during doubled haploid production. The genetic constitution of the diploid donor plant (the chromosomes derived from its parents are indicated by different colours) is rearranged first by intrachromosomal recombination during the (meiotic) pachytene and then subsequently by random chromatid reassortment which is associated with the formation of the haploid megaspores and microspores, the immediate products of female and male meiosis, respectively. These spores are the founder cells of the female (embryo sac) and male (pollen) gametophytes, which themselves give rise to the egg and sperm cells. Whereas the pollen embryogenesis pathway of haploid plant formation involves the induction of cell proliferation and embryogenic development from the microspore or the vegetative cell of a young bicellular pollen, the parthenogenetic route originates either from the egg cell or possibly from another haploid cell type contained in the embryo sac. Haploids produced via uniparental genome elimination genetically derive from an egg or sperm cell, but embryogenic development is triggered by fertilisation, so that this pathway is associated with the transient presence of another haploid set of chromosomes (of *H. bulbosum* in the case of barley) that is lost again during the mitotic divisions taking place in the developing embryo (not shown for simplicity). Once haploid progeny have been successfully regenerated, either spontaneous or artificially induced whole genome duplication results in the restoration of diploidy and ensures that homozygosity prevails at all loci and that the plants are fertile

within the ovule, which forms part of the pistil, while the male gametophytes (pollen) develop within the anther. The result of a normal fertilisation is the fusion of the two gametes to form a diploid zygote, marking the beginning of the sporophytic phase. However, in some wide crosses, one of the two combined parental genomes is eliminated during the first few post-fertilisation mitotic cell divisions, resulting in a haploid embryo; the same process is also documented in intraspecific hybrids when one of the parents is a so-called inducer line (Ravi and Chan 2010). Whereas a zygote is programmed to undergo embryogenesis even

when one of its parental genomes has been lost, gametophytic cells have to be artificially triggered to proliferate, since the normal developmental programme of a gametophyte is strictly determined. The earliest identification of haploid barley plants was made by Johansen (1934), who noted that about 10 % of the seedlings developed from an unnamed cultivar had a distinctive morphology; their cytological behaviour later established that they were haploids. How such individuals arose spontaneously remains unknown.

20.2.1 Uniparental Genome Elimination Following Wide Hybridisation

The reproducible generation of haploid barley plants was first achieved by pollinating barley with *H. bulbosum*. Initial successes were achieved using autotetraploid accessions of both barley and *H. bulbosum* giving rise to dihaploid (diploid) plants (Davies 1958; Kao and Kasha 1969). It was later recognised that even diploid x diploid crosses were effective and that, following in vitro embryo rescue, the regenerated plantlets, which more resembled barley rather than *H. bulbosum* ones, carried just seven chromosomes, the haploid number of barley (Kasha and Kao 1970). Since the cells of developing hybrid embryos harboured up to 11 chromosomes, it was concluded that the formation of a haploid sporophyte relied on the gradual elimination of chromosomes rather than on a parthenogenetic process, as hypothesised by Davies (1958). Kasha and Kao (1970) understood the potential of barley haploids for varietal improvement. Pollination of diploid barley with a tetraploid accession (Lange 1971) or with more distantly related *Hordeum* species was later also shown to induce the formation of haploid embryos; these species include cereal rye (Fedak 1977; Forster and Dale 1983) and maize (Chen et al. 1991), albeit at a significantly lower level of efficiency than with diploid *H. bulbosum*. Surprisingly, perhaps, pollinating either *H. bulbosum* (Lange 1971) or *H. marinum* (Finch 1983; Jorgensen and von Bothmer 1988) with barley generated haploids carrying exclusively the barley nuclear genome, in this case within a non-barley cytoplasm. Ho and Kasha (1975) investigated the genetic control of chromosome elimination by pollinating each of the seven barley trisomic lines with tetraploid *H. bulbosum*, which showed genes on chromosomes 2H and 3H were involved in the elimination. Follow-on experiments based on monotelotrisomic lines indicated that the key genes mapped to both arms of 2H and to the short arm of 3H.

Cytological analyses have established that the full elimination of the *H. bulbosum* chromosomes is completed within nine days (Subrahmanyam and Kasha 1973; Bennett et al. 1976). Sanei et al. (2011) revealed that those *H. bulbosum* chromosomes which do not interact with the mitotic spindle and are therefore not transmitted to the daughter cells appear less condensed and lack a well-defined primary (centromeric) constriction during early mitotic anaphase.

The suggestion was therefore that asynchrony with respect to chromosome condensation must be a major contributor to the loss of chromosomes during cell division; the inability to form a functional kinetochore in a timely manner prevents the normal interaction with the mitotic spindle. Some corroboration has been provided by the observation that the centromeric histone H3 (CENH3) protein, an essential component of the kinetochore and a mediator between the centromere and the spindle microtubules, is present at levels below the detection limit in the centromeres of the lagging chromosomes, even though both *CENH3* mRNA is present and barley CENH3 can be taken up by *H. bulbosum* chromosomes (Sanei et al. 2011). The eventual elimination of the *H. bulbosum* complement is manifested by the formation of micronuclei, which are targeted for degradation. These micronuclei capture either entire or fragmented *H. bulbosum* chromosomes which lag during the separation of the two mitotic daughter cells, while highly condensed chromatin was also observed to be extruded from interphase nuclei (Finch 1983; Gernand et al. 2006).

The elimination process is efficient but not fully effective, as examples of incomplete genome elimination have been observed. Linde-Laursen and von Bothmer (1988) were able to show differences between the seven *H. bulbosum* chromosomes with respect to their propensity to be eliminated. Chromosome elimination is impaired at lower temperatures (Humphreys 1978), with a threshold of 18 °C during the early stages of embryo growth identified by Pickering and Morgan (1985). Whereas the incomplete elimination of the *H. bulbosum* genome can be regarded as a disadvantage in the context of doubled haploid production, the possibility of introgressing genes from *H. bulbosum*, the major representative of the secondary gene pool of cultivated barley, represents a valuable opportunity in barley improvement (Szigat and Pohler 1982; Xu and Kasha 1992). Some *H. vulgare* × *H. bulbosum* crosses have been performed with a view to producing introgression materials (Johnston et al. 2009, see also Chap. 17). Meanwhile, in breeding programmes employing doubled haploid technology, occasional hybrids can usually be recognised and discarded during embryo dissection on the basis of their distinctive shape, and any escapes from this selection step can be picked up at the seedling stage, since the leaves of hybrid plants are covered with soft hairs, a feature which is absent in cultivated barley.

Kasha and Kao (1970) assumed that the propensity to eliminate the *H. bulbosum* genome was genotype independent, but it is now known that some genetically determined variability does exist with respect to both fertilisation success and embryo development. This finding has driven the search for stocks which maximise the yield of haploid embryos (e.g. Simpson et al. 1980; Bjørnstad 1986; Devaux and Pickering 2005). The widespread deployment of the ‘bulbosum’ method during the last two decades of the twentieth century was promoted by the elaboration of robust protocols able to generate a regenerable haploid embryo in up to 30 % of florets pollinated (Kasha and Reinbergs 1976; Jensen 1976; Devaux and Pickering 2005). In a variation of the technique developed by Chen and Hayes (1989), a comparable level of efficiency was achieved by the in vitro culture of newly pollinated florets. Since the 2000s, however, the principle of pollen embryogenesis has largely

replaced uniparental genome elimination as a means of generating barley doubled haploids. The remaining interest in the latter route primarily rests on the finding that segregation bias is more intense in pollen-derived materials than in 'bulbosum'-derived ones (Devaux et al. 1995; Sayed et al. 2002; Cistué et al. 2011); this is of particular importance in the development of mapping populations (Johnston et al. 2009).

20.2.2 Parthenogenesis

Haploid plant formation is promoted in the *hap* mutant, which was induced by ethyl methane sulphonate treatment (Hagberg and Hagberg 1980). Up to 40 % of the embryos produced by self-pollinating a plant homozygous for the mutant allele are haploid progeny, while this frequency drops to 1–7 % in *hap* heterozygotes (Hagberg et al. 1985). The selfed progeny of a wild type x *hap* mutant hybrid include a significant proportion of haploid individuals, which implies that the genes underlying the trait are nuclear rather than cytoplasmic. A transmission electron microscopy-based investigation of the process of haploid embryo formation in the *hap* mutant has shown that the key event is the failure of fusion of the egg and sperm cell, even though the endosperm arises and develops normally (Mogensen 1982). What remains obscure is how the unfertilised *hap* egg cell is triggered to undergo embryogenesis. A scheme for exploiting the mutant for producing doubled haploid barley was elaborated by Hagberg et al. (1985). Its basis rested on the ability of *hap* heterozygotes to produce haploid progeny which harboured the wild type rather than the *hap* allele and further relied on the idea to develop a facile marker for these progeny (Hagberg and Hagberg 1987). The system has not so far been incorporated into a practical breeding approach, likely because of the low rate of haploid formation from *hap* heterozygotes and the risk of linkage drag associated with the mutant allele.

The successful regeneration of putative haploid barley plants from an unpollinated pistil explant cultivated in vitro has been reported on several occasions (San Noeum 1976; Wang and Kuang 1981; Huang et al. 1982; Castillo and Cistué 1993). A disadvantage of this approach is that it is difficult to exclude the products of accidental self-pollination, and furthermore none of these studies provided compelling evidence that the regenerants originate from the egg cell or another gametophytic cell type. Although an efficiency of up to ten plants per hundred cultured pistils has been reported, there are surprisingly no published examples of the use of this approach in either biotechnology or plant breeding practice.

20.2.3 Pollen Embryogenesis

The currently most widely employed method for producing barley haploids is based on the capacity to induce immature pollen to undergo cell proliferation and become embryogenic, a developmental route which has never been observed to occur naturally. The reprogramming of pollen development requires the imposition of a stress episode to abolish the cell's normal identity, followed by a period of culture in a medium which promotes cell proliferation and subsequent embryogenesis. Various stress conditions are effective, amongst which the commonest employed are low or high temperature and nutritional deficiency. The stress can be imposed either on the intact spike (Mordhorst and Lörz 1993), on dissected anthers (Roberts-Oehlschlager and Dunwell 1990; Hoekstra et al. 1992) or on isolated pollen (Kumlehn and Lörz 1999). Combinations of stress treatments have been reported to overcome the refractoriness of some cultivars (Coronado et al. 2005). Some anther culture protocols include no specific stress treatment but are nevertheless likely to expose the pollen to starvation given the temporary interruption in the supply of nutrients after anther dissection resulting from the time needed for components of the culture medium to diffuse through the anther wall. The developmental stage during which barley pollen is most readily triggered into embryogenic growth is around pollen mitosis I, between the time when the microspores are fully vacuolated (premitotic) and bicellular pollen grains were just formed. The physiological switch to amyloplast formation and starch accumulation which takes place in bicellular pollen grains is thought to be closely associated with the loss of the pollen's capacity of diverting its development away from its normal course towards cell proliferation and embryogenic growth (Daghma et al. 2014).

The formation of multicellular pollen structures can be achieved either by culturing an intact anther or by first releasing the immature pollen into the culture medium—both approaches are employed. Clapham (1973) was the first to describe the production of haploid barley plants following the pollen embryogenesis pathway, but at that time efficiency levels were low and a significant proportion of the regenerants lacked chlorophyll. Substantial progress has been achieved since this time by the use of mannitol solutions to expose spikes or anthers to carbohydrate starvation under appropriate osmotic conditions (Roberts-Oehlschlager and Dunwell 1990; Kasha et al. 2001), by optimising the severity of inductive stress (e.g. Coronado et al. 2005) and by developing protocols involving embryogenic pollen released at an early timepoint from anthers cultivated in liquid medium or by the isolation of immature pollen prior to cultivation (Ziauddin et al. 1990; Hoekstra et al. 1992). Major improvements of nutrient media for anthers or isolated pollen were associated with the use of maltose as a source of slowly accessible carbohydrate during embryogenic growth (Scott et al. 1995; Hunter 1989) and the use of significantly less ammonium as compared to the standard MS or B5 media (Mordhorst and Lörz 1993; Murashige and Skoog 1962; Gamborg et al. 1968). The capacity to regenerate plants from pollen-derived tissue has also been improved, e.g. by bringing forward the transfer of multicellular pollen structures

onto a solid medium and by increasing the concentration of copper sulphate in the medium (Kumlehn et al. 2006).

Further improvements in the efficiency of pollen embryogenesis have been hampered by a poor understanding of the underlying biological processes. A descriptive histological investigation based on fixed pollen structures was first conducted by Sunderland et al. (1979) and later extended by Ramírez et al. (2001). Meanwhile, Kumlehn and Lörz (1999) and Maraschin et al. (2005) tracked the development of living individual pollen grains by first immobilising them. The level of detail was limited by the length of the time interval between consecutive observations. Better definition has been achieved more recently by monitoring at 3 min intervals the development of vacuolated premitotic microspores up to the formation of multicellular, actively growing pollen structures (Daghma et al. 2012). Nine embryogenic and non-embryogenic types of pollen response to culture conditions were recognised. In the major embryogenic pathway, cell proliferation started from a symmetric mitosis (>50 % of pollen structures), whereas an asymmetric mitosis was associated with embryogenic development in less than 5 % of the test pollen. In the latter case, proliferation generally originated from a vegetative-like cell, while the generative-like one did not contribute to embryogenic development. Although generative-like cells occasionally divided, no further cell proliferation ensued (Daghma et al. 2014). In the same study, there was also evidence that the nuclear fusion of mitosis-derived pairs of daughter nuclei is the essential event for spontaneous whole genome doubling during barley pollen embryogenesis. Nuclear fusion events were observed throughout the process of pollen embryogenesis, explaining the known chimeric ploidy of microcalli and regenerants. The observations are consistent with those previously obtained using electron microscopy which have suggested that when nuclei coexist within a single cytoplasm following incomplete cytokinesis, their envelopes may fuse (González-Melendi et al. 2005).

The advent of 'omics' technologies has clarified some of the cellular processes associated with the initiation of pollen embryogenesis (Hosp et al. 2007). In barley, both *ECLTP* and *ECA1* are upregulated in early embryogenic pollen cultures (Vrinten et al. 1999); the former one shares sequence homology with a gene associated with embryogenic clusters in carrot cell cultures (Sterk et al. 1991), while an orthologue of the latter is highly activated in wheat egg cells (Sprunck et al. 2005). Nevertheless the product of neither of the two genes is likely to act as a sufficient trigger of the developmental switch from gametophytic to embryogenic pollen development. Other transcriptomic data sets have revealed at best genes playing a role in the cellular response to stress or genes which are in some way associated with embryogenesis rather than being essential factors for its initiation (Maraschin et al. 2006; Muñoz-Amatriaín et al. 2009). The major challenge which remains is that most of the cells present within an embryogenic pollen culture do

not differentiate into regenerable structures; rather they undergo programmed cell death. Consequently, there is a major risk of incorrect identification of genes, proteins and metabolites specifically associated with pollen embryogenesis.

20.2.4 Whole Genome Duplication

The frequency of spontaneous whole genome duplication in the product of an interspecific cross in which one parental genome has been eliminated is typically low, so progeny are mostly haploid. When both parental plants are auto polyploid, the resulting plants are typically, as expected, polyhaploid, yet those individuals are unlikely to be homozygous and thus will not be particularly useful for breeding purposes. In contrast, the majority of barley plants regenerated from embryogenic pollen cultures do experience whole genome duplication, as evidenced by the fact that at least some of their florets are self-fertile. Nonetheless, an artificial triggering of whole genome duplication can be desirable, e.g. when only few plants can be produced for some reason (Kumlehn et al. 2006). Colchicine-based whole genome duplication protocols, the use of which results in 50–80 % of doubled haploid regenerants, have been established for many years (e.g. Thiebaut and Kasha 1978), and no significant improvements in efficiency have been reported in the intervening years.

20.2.5 Evidence of the Cellular Origin and for the Homozygosity of Regenerants

Plants regenerated from cultured gametophytic explant material have not necessarily developed from a haploid cell, since the gametophyte can harbour some diploid cells following meiotic restitution events (Ramanna 1979; Bretagnolle and Thompson 1995) and also adjacent somatic (maternal) tissues may give rise to regenerants (Munyon et al. 1989; Arzate-Fernández et al. 1997; Bal et al. 2012). A thorough validation of a doubled haploid protocol is therefore needed before it can be extended to large-scale production. In some species, the homozygosity of doubled haploids has been confirmed using a variety of DNA-based markers (Meyer et al. 1993; Chani et al. 2000; Murovec et al. 2007; Nelson et al. 2009). More recently, Hofinger et al. (2013) have exploited to good effect an enzymatic mismatch cleavage assay to screen parental, F_1 hybrid and putative doubled haploids in barley.

20.3 Applications

20.3.1 Research and Pre-breeding

Populations of doubled haploids have been widely used to analyse the inheritance of quantitative traits, to produce genetic maps and to derive marker-trait associations (see also Chaps. 1, 15, 16, 18 and 19). Regenerable haploid cells are a particularly attractive target for recovering induced mutants, since it has the effect of fixing the mutated allele via whole genome duplication. The induction of mutants by irradiating barley spikes with γ-rays prior to anther dissection and culture has been reported by Szarejko et al. (1995). Castillo et al. (2001) mutagenised immature anthers and isolated microspores with sodium azide. Amongst the regenerants following pollen embryogenesis, some 15 % were reported to be morphologically altered in heritable fashion. Likewise, embryogenic pollen cultures have been successfully genetically transformed using both an *Agrobacterium*-mediated and a biolistics-based approach (Kumlehn et al. 2006; Shim et al. 2009). Kapusi et al. (2013) took advantage of the simplified segregation behaviour of doubled haploids (compared to that shown by sexually generated progeny) to produce selectable marker-free transgenic individuals. More recently, Gurushidze et al. (2014) have described a means to efficiently generate site-directed, true-breeding gene knockouts in barley using a customised transcription activator-like endonuclease. For more details on the synergy between haploid technology and transgenesis, see Chap. 21.

20.3.2 Practical Breeding

Haploid technology can enhance the efficiency of selection with regard to quantitative traits because it avoids the problem of genetic heterogeneity which is a feature of conventionally produced early generation material. It allows the bringing forward to a very early stage in the breeding programme of selection for traits which require replicated trialling—these traits are typically not testable before the F_5 generation in a pedigree-based conventional programme. The resulting savings in time and field resources can be substantial. The downside of the doubled haploid approach is the effort required to produce large numbers of progeny, which is a facile matter in conventional breeding programmes. Most current barley breeding programmes, at least in Europe, employ doubled haploid technology (see also Chap. 1). Six-rowed winter barley F_1 hybrid cultivars, which provide higher levels of yield and yield stability than conventional inbred cultivars (Mühleisen et al. 2014), are enjoying a steady increase in market share in Europe. An essential component of F_1 hybrid breeding is the maintenance of true-breeding parental lines, and doubled haploid technology provides a robust means to produce such materials.

Conclusion and Perspective

The key molecular mechanisms which trigger pollen embryogenesis and uniparental genome elimination—the two major props of haploid technology—are still obscure. Barley is a particularly useful experimental model for elucidating these mechanisms, since it enjoys a wealth of genetic and genomic resources, and is at the forefront of transgenic and mutation research. With respect to pollen embryogenesis, the stress treatments required to derail the normal developmental path in order to promote cell proliferation imply that the necessary cellular state lies on a knife edge between survival and death, and this perhaps reflects the continuing low frequency of embryo production achieved even from amenable genotypes. It is conceivable that the highest level of nonfatal stress which can be imposed may still be insufficient to induce embryogenic development in the most refractory cultivars. Since fertilisation success rates in interspecific crosses tend to be lower than in intraspecific ones, it would be advantageous to develop haploid inducer barley lines. The recognition of the CENH3 histone as a key determinant of the interaction between chromosomes and the mitotic spindle may pave the way towards this goal (Chan 2011). The greater our understanding of the cellular and molecular basis of haploid formation, the more feasible it will become to induce haploids under less stressful conditions than are utilised in current protocols, which should improve the efficiency of haploid plant production and avoid the problem of genotype dependency. Haploid technology already makes a major contribution to modern barley breeding but in addition has utility in the areas of marker discovery, genetic mapping, introgression breeding, mutagenesis and transgenesis. As such, its continuing use into the future seems to be assured.

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References

Arzate-Fernández A-M, Nakazaki T, Okumoto Y, Tanisaka T (1997) Efficient callus induction and plant regeneration from filaments with anther in lily (Lilium longiflorum Thunb.). Plant Cell Rep 16:836–840

Bal U, Shariatpanahi ME, Castro AJ, Emery D, Clément C, Dehestani-Ardakani M, Mozaffari K, Touraev A (2012) Pseudo-embryogenic structures in anther and isolated microspore cultures in vitro: a cautionary guide. Czech J Genet Plant Breed 48:51–60

Bennett MD, Barclay IR, Finch RA (1976) The time rate and mechanism of chromosome elimination in Hordeum hybrids. Chromosoma 54:175–200

Bjørnstad A (1986) Partial incompatibility between Scandinavian six-rowed barleys (Hordeum vulgare L.) and Hordeum bulbosum L., and its genetical basis. Hereditas 104:171–191

Bretagnolle F, Thompson JD (1995) Tansley review no. 78. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol 129:1–22

Castillo AM, Cistué L (1993) Production of gynogenic haploids of Hordeum vulgare L. Plant Cell Rep 12:139–143

Castillo AM, Cistué L, Vallés MP, Sanz JM, Romagosa I, Molina-Cano JL (2001) Efficient production of androgenic doubled-haploid mutants in barley by the application of sodium azide to anther and microspore cultures. Plant Cell Rep 20:105–111

Chan SWL (2011) In a battle between parental chromosomes, a failure to reload. Proc Natl Acad Sci U S A 108:13361–13362

Chani E, Veilleux RE, Boluarte-Medina T (2000) Improved androgenesis of interspecific potato and efficiency of SSR markers to identify homozygous regenerants. Plant Cell Tiss Organ Cult 60:101–112

Chen FQ, Hayes PM (1989) A comparison of Hordeum bulbosum-mediated haploid production efficiency in barley using in vitro floret and tiller culture. Theor Appl Genet 77:701–704

Chen FQ, Hayes PM, Rivin CJ (1991) Wide hybridization of Hordeum vulgare and Zea mays. Genome 34:603–605

Cistué L, Cuesta-Marcos A, Chao S, Echávarri B, Chutimanitsakun Y, Corey A, Filichkina T, Garcia-Marino N, Romagosa I, Hayes PM (2011) Comparative mapping of the Oregon Wolfe barley using doubled haploid lines derived from female and male gametes. Theor Appl Genet 122:1399–1410

Clapham D (1973) Haploid Hordeum plants from anthers in vitro. Z Pflanzenzucht 69:142–155

Coronado MJ, Hensel G, Broeders S, Otto I, Kumlehn J (2005) Immature pollen-derived doubled haploid formation in barley cv. Golden Promise as a tool for transgene recombination. Acta Physiol Plant 27:591–599

Daghma DS, Kumlehn J, Hensel G, Rutten T, Melzer M (2012) Time-lapse imaging of the initiation of pollen embryogenesis in barley (Hordeum vulgare L.). J Exp Bot 63:6017–6021

Daghma DS, Hensel G, Rutten T, Melzer M, Kumlehn J (2014) Cellular dynamics during early barley pollen embryogenesis revealed by time-lapse imaging (submitted)

Davies DR (1958) Male parthenogenesis in barley. Heredity 12:493–498

Devaux P, Pickering R (2005) Haploids in the improvement of Poaceae. In: Palmer CE, Kasha KJ, Keller WA (eds) Haploids in crop improvement II, vol 56, Biotechnology in agriculture and forestry. Springer, Berlin, pp 215–242

Devaux P, Kilian A, Kleinhofs A (1995) Comparative mapping of the barley genome with male and female recombination-derived, doubled haploid populations. Mol Gen Genet 249:600–608

Fedak G (1977) Haploids from barley × rye crosses. Can J Genet Cytol 19:15–19

Finch RA (1983) Tissue-specific elimination of alternative whole parental genomes in one barley hybrid. Chromosoma 88:386–393

Forster BP, Dale JE (1983) Effects of parental embryo and endosperm mitotic cycle times on development of hybrids between barley and rye. Ann Bot 52:613–620

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gernand D, Rutten T, Pickering R, Houben A (2006) Elimination of chromosomes in Hordeum vulgare x H. bulbosum crosses at mitosis and interphase involves micronucleus formation and progressive heterochromatinization. Cytogenet Genome Res 114:169–174

González-Melendi P, Ramírez C, Testillano PS, Kumlehn J, Risueno MC (2005) Three dimensional confocal and electron microscopy imaging define the dynamics and mechanisms of diploidisation at early stages of barley microspore-derived embryogenesis. Planta 222:47–57

Gurushidze M, Hensel G, Hiekel S, Schedel S, Valkov V, Kumlehn J (2014) True-breeding targeted gene knock-out in barley using designer TALE-nuclease in haploid cells. PLoS One 9(3):e92046

Hagberg A, Hagberg G (1980) High frequency of spontaneous haploids in the progeny of an induced mutation in barley. Hereditas 93:341–343

Hagberg A, Hagberg G (1987) Production of spontaneously doubled haploids in barley using a breeding system with marker genes and the “hap” gene. Biol Zentralbl 106:5358

Hagberg G, Hagberg A, Heneen WK (1985) Haploidy initiator gene, “hap”, in barley. In: Proceedings of 1st Nordic cell and tissue culture symposium on research, breeding and production of crop plants, Frostavallen, Sweden, 5–9 March 1985 (Hereditas Suppl vol 3)

Ho KM, Kasha KJ (1975) Genetic control of chromosome elimination during haploid formation in barley. Genetics 81:263–275

Hoekstra S, van Zijdeveld MH, Louwerse JD, Heidekamp F, van der Mark F (1992) Anther and microspore culture of Hordeum vulgare L. cv. Igri. Plant Sci 86:89–96

Hofinger BJ, Huynh OA, Jankowicz-Cieslak J, Müller A, Otto I, Kumlehn J, Till BJ (2013) Validation of doubled haploid plants by enzymatic mismatch cleavage. Plant Methods 9:43

Hosp J, Maraschin SF, Touraev A, Boutilier K (2007) Functional genomics of microspore embryogenesis. Euphytica 158:275–285

Huang QT, Yang HY, Zhou C (1982) Embryological observations on ovary culture of unpollinated young flowers in Hordeum vulgare L. Acta Bot Sin 24:295–300

Humphreys MW (1978) Chromosome instability in Hordeum vulgare x H. bulbosum hybrids. Chromosoma 65:301–307

Hunter CP (1989) US Patent 4,840,906 Plant generation method

Jensen CJ (1976) Barley monoploids and doubled monoploids: techniques and experience. In: Gaul H (ed) Proceedings of III international barley genetics symposium, Garching, 1975. Verlag Karl Thiemig, München, pp 316–345

Johansen DA (1934) Haploids in Hordeum vulgare. Proc Natl Acad Sci U S A 20:98–100

Johnston PA, Farnden KJ, Pickering R, Timmerman-Vaughan GM (2009) Marker development and characterisation of Hordeum bulbosum introgression lines: a resource for barley improvement. Theor Appl Genet 118:1429–1437

Jorgensen RB, von Bothmer R (1988) Haploids of Hordeum vulgare and H. marinum from crosses between the two species. Hereditas 108:207–212

Kao KN, Kasha KJ (1969) Haploidy from interspecific crosses with tetraploid barley. In: Nilan RA (ed) Proceedings of 2nd international barley genetics symposium, 6–11 July 1969. Washington State University Press, Washington, pp 82–88

Kapusi E, Hensel G, Coronado M-J, Broeders S, Marthe C, Otto I, Kumlehn J (2013) The elimination of a selectable marker gene in the doubled haploid progeny of co-transformed barley plants. Plant Mol Biol 81:149–160

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (Hordeum vulgare L.). Nature 225:874–876

Kasha KJ, Reinbergs E (1976) Haploidy and polyploidy and its application in breeding techniques. In: Gaul H (ed) Proceedings of III international barley genetics symposium, Garching, 1975. Verlag Karl Thiemig, München, pp 307–315

Kasha KJ, Hu TC, Oro R, Simion E, Shim YS (2001) Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (Hordeum vulgare L.) microspores. J Exp Bot 52:1227–1238

Kumlehn J, Lörz H (1999) Monitoring sporophytic development of individual microspores of barley (Hordeum vulgare L.). In: Clement C, Pacini E, Audran J-C (eds) Anther and pollen. Biology to biotechnology. Springer, Berlin, pp 183–190

Kumlehn J, Serazetdinova L, Hensel G, Becker D, Lörz H (2006) Genetic transformation of barley (Hordeum vulgare L.) via infection of androgenetic pollen cultures with Agrobacterium tumefaciens. Plant Biotechnol J 4:251–261

Lange W (1971) Crosses between Hordeum vulgare L and H. bulbosum L.1. Production morphology and meiosis of hybrids, haploids and dihaploids. Euphytica 20:14–29

Linde-Laursen I, Von Bothmer R (1988) Elimination and duplication of particular Hordeum vulgare chromosomes in aneuploid interspecific Hordeum hybrids. Theor Appl Genet 76:897–908

Maraschin SF, Vennik M, Lamers GEM, Spaink HP, Wang M (2005) Time-lapse tracking of barley androgenesis reveals position-determined cell death within pro-embryos. Planta 220:531–540

Maraschin SF, Caspers M, Potokina E, Wülfert F, Graner A, Spaink HP, Wang M (2006) cDNA array analysis of stress-induced gene expression in barley androgenesis. Physiol Plant 127:535–550

Meyer R, Salamini F, Uhrig H (1993) Isolation and characterization of potato diploid clones generating a high frequency of monohaploid or homozygous diploid androgenetic plants. Theor Appl Genet 85:905–912

Mogensen L (1982) Double fertilization in barley and the cytological explanation for haploid embryo formation, embryoless caryopses, and ovule abortion. Carlsberg Res Commun 47:313–354

Mordhorst AP, Lörz H (1993) Embryogenesis and development of isolated barley (Hordeum vulgare L.) microspores are influenced by the amount and composition of nitrogen sources in culture media. J Plant Physiol 142:485–492

Mühleisen J, Piepho H-P, Maurer HP, Longin CFH, Reif JC (2014) Yield stability of hybrids versus lines in wheat, barley, and triticale. Theor Appl Genet 127:309–316

Muñoz-Amatriaín M, Svensson JT, Castillo AM, Close TJ, Vallés MP (2009) Microspore embryogenesis: assignment of genes to embryo formation and green vs. albino plant production. Funct Integr Genomics 9:311–323

Munyon IP, Hubstenberger JF, Phillips GC (1989) Origin of plantlets and callus obtained from chile pepper anther cultures. In Vitro Cell Dev Biol 25:293–296

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murovec J, Stajner N, Jakse J, Javornik B (2007) Microsatellite marker for homozygosity testing of putative doubled haploids and characterization of mimulus species derived by a cross-genera approach. J Am Soc Hortic Sci 132:659–663

Nelson MN, Mason AS, Castello M-C, Thomson L, Yan G, Cowling WA (2009) Microspore culture preferentially selects unreduced (2n) gametes from an interspecific hybrid of Brassica napus L. x Brassica carinata Braun. Theor Appl Genet 119:497–505

Pickering RA, Morgan PW (1985) The influence of temperature on chromosome elimination during embryo development in crosses involving Hordeum spp, wheat (Triticum aestivum L) and rye (Secale cereale L.). Theor Appl Genet 70:199–206

Ramanna MS (1979) A re-examination of the mechanisms of 2n gamete formation in potato and its implications for breeding. Euphytica 28(537):561

Ramírez C, Testillano PS, Castillo A-M, Vallés M-P, Coronado M-J, Cistué L, Risueño MC (2001) The early microspore embryogenesis pathway in barley is accompanied by concrete ultrastructural and expression changes. Int J Dev Biol 45(S1):57–58

Ravi M, Chan SWL (2010) Haploid plants produced by centromere-mediated genome elimination. Nature 464:615–619

Roberts-Oehlschlager SL, Dunwell JM (1990) Barley anther culture: pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell Tiss Organ Cult 20:235–240

San Noeum LH (1976) Haploides d'Hordeum vulgare L. par culture in vitro d'ovaries non fecondes. Ann Amelior Plant 26:751–754

Sanei M, Pickering R, Kumke K, Nasuda S, Houben A (2011) Loss of centromeric histone H3 (CENH3) from centromeres precedes uniparental chromosome elimination in interspecific barley hybrids. Proc Natl Acad Sci U S A 108:498–505

Sayed H, Kayyal H, Ramsey L, Ceccarelli S, Baum M (2002) Segregation distortion in doubled haploid lines of barley (Hordeum vulgare L.) detected by simple sequence repeat (SSR) markers. Euphytica 225:265–272

Scott P, Lyne RL, Rees T (1995) Metabolism of maltose and sucrose by microspores isolated from barley (Hordeum vulgare L.). Planta 197:435–441

Shim Y-S, Pauls KP, Kasha KJ (2009) Transformation of isolated barley (Hordeum vulgare L.) microspores. II. Timing of pretreatment and temperatures relative to results of bombardment. Genome 52:175–190

Simpson E, Snape JW, Finch RA (1980) Variation between Hordeum bulbosum genotypes in their ability to produce haploids of barley, Hordeum vulgare. Z Pflanzenzucht 85:205–211

Sprunck S, Baumann U, Edwards K, Langridge P, Dresselhaus T (2005) The transcript composition of egg cells changes significantly following fertilization in wheat (Triticum aestivum L.). Plant J 41:660–672

Sterk P, Booij H, Schellekens GA, van Kammen A, de Vries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3:907–921

Subrahmanyam N, Kasha K (1973) Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. Chromosoma 42:111–125

Sunderland N, Roberts M, Evans LJ, Wildon DC (1979) Multicellular pollen formation in cultured barley anthers. I. Independent division of the generative and vegetative cells. J Exp Bot 30:1133–1144

Szarejko I, Guzy J, Davalos JJ, Chavez AR, Maluszynski M (1995) Production of mutants using barley DH systems. Induced mutations and molecular techniques for crop improvement. IAEA, Vienna, pp 517–530

Szigat G, Pohler W (1982) Hordeum bulbosum x H. vulgare hybrids and their backcrosses with cultivated barley. Cereal Res Commun 10:73–78

Thiebaut J, Kasha KJ (1978) Modification of the colchicine technique for chromosome doubling of barley haploids. Can J Genet Cytol 20:513–521

Vrinten PL, Nakamura T, Kasha KJ (1999) Characterization of cDNAs expressed in the early stages of microspore embryogenesis in barley (Hordeum vulgare L.). Plant Mol Biol 41:455–463

Wang CC, Kuang BG (1981) Induction of haploid plants from the female gametophyte of Hordeum vulgare L. Acta Bot Sin 23:329–330

Xu J, Kasha KJ (1992) Transfer of a dominant gene for powdery mildew resistance and DNA from Hordeum bulbosum into cultivated barley (H. vulgare). Theor Appl Genet 84:771–777

Ziauddin A, Simion E, Kasha KJ (1990) Improved plant regeneration from shed microspore culture in barley (Hordeum vulgare L.) cv. Igri. Plant Cell Rep 9:69–72

Chapter 21
Genetic Engineering

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21.1 Introduction

Barley is not just the leading experimental model for the temperate small grain cereals but is a major crop in its own right. The development of transgenic technology has opened the way to both generating novel genetic solutions relevant to crop improvement and to the routine validation of gene function. The recent acquisition of comprehensive barley genome sequence data has fuelled a major effort into defining the role of a whole spectrum of genes, some of which have been recognized for many years and others which are only now being identified. This chapter describes the current state of the art in barley transgenesis and the major principles of genetic engineering in the species, while other chapters in this volume address specific applications of barley transformation technology. Besides providing a historical background and a description of well-established methodologies, this review also touches on newly emerging approaches such as host-induced gene silencing as well as chromosome and genome engineering.

21.2 Gene Transfer

Recombinant DNA can be introduced into barley cells either via direct gene transfer, in which extracted plasmid DNA is used, or via infection by a virus or the plant bacterial pathogen *Agrobacterium tumefaciens*. The host cell may be either transiently or stably transformed; to assure the latter, the transgene needs to be integrated into the barley genome, as otherwise it will be lost in the course of

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further development. Successful transformation also relies on a judicious choice of host recipient cells or tissues. Most transgenic experiments choose to include a marker gene which allows for the straightforward selection of transformed cells through the application of a relevant selective pressure (e.g. the incorporation of an herbicide or an antibiotic into the culture medium).

21.2.1 Transient Expression Systems

Early transformation experiments focused on the detection of the transient expression of a reporter gene (such as *GUS*, the gene encoding β-glucuronidase). Numerous studies were aimed at maximizing the level of transient expression, with a view to establishing the most suitable conditions required to achieve stable transformation and the generation of transgenic plants. Some of these attempts employed either electric fields or specific chemicals to induce suspended host cells or protoplasts to accept foreign DNA [for a review, see Goedeke et al. (2007)]. Later, the biolistic method was perfected, in which metal particles coated with a plasmid harbouring the transgene were fired into the host explant. This method proved to be an effective means of validating gene function, particularly because, typically, multiple copies of the transgene were introduced and any tissue accessible to the bombardment could be selected as the target. These advantages ensured that the process of screening candidate DNA sequences was much more time efficient than the more laborious process of generating stable transformants. The format of the experiment allowed for combinations of expression cassettes to be included in a single bombardment, and this feature was particularly useful as it could be exploited, by including a reporter gene such as *GUS*, as a simple means of indicating in which individual cells the transformation had been successful. The establishment of the Gateway cloning method (Himmelbach et al. 2007) and automated microscopic screens (Douchkov et al. 2005; Ihlow et al. 2008) has further facilitated the use of large-scale reverse genetic screens (see also Chap. 11), whether to check for over-expression, the RNA interference-mediated knock-down of gene expression or host-induced silencing of pathogen genes (see also below). Examples in barley have included the analysis of the plant-pathogen interaction in the leaf epidermis (Zimmermann et al. 2006; Delventhal et al. 2011) and the assessment of gene function in the response to dehydration (Marzin et al. 2008).

Some RNA viruses have been shown to act as effective transgene vectors for the purpose of transient expression. Virus-based expression systems exploit the movement of pathogen far beyond the initial infection site, as well as the high transgene copy number which is achieved as a result of the virus' replication in planta and associated with substantial levels of transgene expression. An example of this approach was described by Choi et al. (2000), who demonstrated high levels of transgene expression in barley via the infection of a derivative of the wheat streak mosaic virus. However, virus-based expression systems are used for gene

knock-down rather than over-expression, because the replication of the virus is frequently accompanied by sequence-specific silencing in which the host target gene can be co-suppressed. A further limitation on the use of this approach is that the consistent infection of the host can sometimes be difficult to ensure, while in addition, there is a limit over the size of the transgene itself, since long sequences compromise the stability of the vector. Although at one time the brome mosaic virus was seen as a viable vector (French et al. 1986; Ding et al. 2006), the bulk of virus-induced gene silencing in barley has focused on barley stripe mosaic virus (BSMV) (Holzberg et al. 2002; Oikawa et al. 2007). Steady technical improvements to the BSMV-based method have turned it into a powerful reverse genetic tool in barley; further improvements of this approach have been to use cDNA-based constructs rather than relying on RNA obtained via in vitro transcription, to use a customized vector allowing for the straightforward insertion of DNA fragments, to deliver the vector via biolistics or agroinoculation rather than by the less consistent expedient of wounding and to use *Nicotiana benthamiana* leaf material as a particularly rich source of virus (Meng et al. 2009; see also Chap. 11). Both the cellular and organismal response to the transgenic state cannot be decoupled from the potentially negative impact of the transformation procedure itself. Furthermore, the high gene dosages often induced in transient expression formats can in themselves be detrimental to growth and survival. Thus, the data generated in these experiments need to be interpreted with some care and ideally should be substantiated by complementary methodologies.

21.2.2 Stable Transgenic Systems

The production of stable transgenic plants requires that the transgene be integrated into the host's genome. In order to be transmitted to the next sexual generation, the integration must involve cells contributing to the formation of the gametophyte. The generation of stable transgenic barley plants was first demonstrated by the biolistic transfer of plasmid DNA into protoplasts isolated from a variety of explant material, followed by the regrowth of the cell wall, cell proliferation and finally differentiation into a plant (Lazzeri et al. 1991; Funatsuki et al. 1995; Salmenkallio-Marttila et al. 1995; Nobre et al. 2000). These early experiments suffered from too low a transformation efficiency to be useful as a routine application. Attempts to microinject plasmid DNA into isolated (protoplast-like) zygotes did not achieve much success in producing plants in which the transgene was expressed, as genomic integration was largely confined to nonfunctional transgene fragments (Holm et al. 2000). None of these experiments demonstrated the generative transmission of the transgene, and the use of these methods was limited to showing that reporter genes could be expressed successfully; in part, the lack of positive results reflects the technical challenges of handling barley protoplasts and regenerating plants from them (Stöldt et al. 1996). When cultured seedling shoot meristem explants were used by Zhang et al. (1999) as the explant for biolistics-based transgenesis, some

primary transgenic plants were regenerated, but an analysis of their genomic DNA indicated that all of the regenerants must have originated from a single transformation event. The literature does not include any further explorations of this approach. The most successful early technique for obtaining stably transformed barley relied on biolistics-based transfer to immature embryos (Wan and Lemaux 1994), and this method proved to be valuable in a number of follow-up studies [as examples, see Williams-Carrier et al. (1997) and Cho et al. (1999)].

Although initial efforts to establish stable transgenesis using *Agrobacterium* were circumscribed by a poor level of compatibility between *A. tumefaciens* and monocotyledonous species, by the late 1990s, the barley biolistics platform was largely replaced by *Agrobacterium*-mediated transformation. The first stable transgenic barley achieved using *Agrobacterium* (Tingay et al. 1997) combined several measures considered at the time to be rather unconventional: firstly, a highly stable binary vector, taking advantage of the RK2 origin of replication, was used; secondly, promoters driving strong expression in monocotyledonous hosts were included; thirdly, a hypervirulent *A. tumefaciens* strain (AGL1) was chosen; fourthly, the bacterial inoculum was grown in the absence of any antibiotics in order to minimize damage to the cocultivated plant cells; fifthly, after coculture, the immature embryos were placed onto medium with their scutellum facing down; and finally, the highly effective antibiotic Timentin was used to rapidly kill off the *Agrobacterium* cells following the coculture phase. These principles still underlie current standard barley transformation technology (Bartlett et al. 2008; Hensel et al. 2009). Additional improvements have incorporated acetosyringone to trigger the *A. tumefaciens* cells' transformation machinery and the *E. coli HPT* gene (encoding hygromycin phosphotransferase) as an efficient selectable marker in conjunction with the use of hygromycin as the agent to inhibit the development of non-transformed plant cells (Horvath et al. 2000; Matthews et al. 2001). Carrying out the explant inoculation step in a liquid medium has greatly increased the throughput, while the inclusion of cysteine in the cocultivation medium has materially improved transformation efficiency, thanks to its mitigation of the host cells' wound- and pathogen-induced response (Hensel et al. 2008). Genotype dependency with respect to transformability remains an issue (Murray et al. 2004; Hensel et al. 2008), although its genetic basis is still obscure. Thus, the standard barley transformation method still relies on cv. 'Golden Promise', whose usefulness for stable transgenesis is reflected by numerous research reports (for review, see Kumlehn et al. (2010); see also Chaps. 3–13). A recently developed doubled haploid selection termed 'Golden SusPtrit' combines extreme susceptibility to the rust pathogen *Puccinia tritici* with a level of transformability on a par with that of cv. 'Golden Promise' (Yeo et al. 2014). This experimental line was developed to facilitate studies on the interaction of barley with fungal pathogens.

Notwithstanding the successful use of immature embryos as the explant for biolistics-based and *Agrobacterium*-mediated transformation, other targets have also been explored, given that they have some specific advantages. The poor transformability of many cultivars was addressed by a demonstration that plant regeneration from an explant consisting of the ovule isolated shortly after

fertilization was largely genotype independent (Holme et al. 2006, 2008). A range of cultivars was shown to be transformable via this route, although the level of transformation efficiency achieved in other cultivars remained consistently below that of 'Golden Promise'. A particularly attractive target is the (haploid) gametophyte, since the whole-genome doubling of a resultant haploid transgenic plant instantly fixes the transgene in the homozygous state (as well as restoring fertility). Both biolistics-based (Jähne et al. 1994; Carlson et al. 2001) and agroinoculation-based (Kumlehn et al. 2006) attempts to transform immature pollen primed to undergo embryogenic development rather than to complete the pollen maturation process have been described. Embryogenic pollen cultures have proven to be rather sensitive to external stress, so are not easy to manage. Nevertheless, the approach has resulted in a reproducible means of instantly generating homozygous transgenic barley (Kumlehn et al. 2006; Shim et al. 2009; Gurushidze et al. 2014). The agroinoculation of embryogenic pollen cultures has become the method of choice for transforming winter barley and has been used in experiments both aimed at the functional analysis of transgenes (Stein et al. 2005; Radchuk et al. 2006) and at the establishment of site-directed mutagenesis technology based on novel endonucleases (Gurushidze et al. 2014; see also below).

A key element in the production of transgenic materials is the effectiveness of the selective agent used to suppress the growth of non-transformed cells. Early attempts to transform isolated protoplasts relied on the presence of the *E. coli NPTII* gene, which confers resistance to kanamycin (Lazzeri et al. 1991; Funatsuki et al. 1995). The *Streptomyces*-derived *PAT* or *BAR* genes, which determine tolerance to the herbicide phosphinothricin and its derivatives, proved to be more efficacious (Wan and Lemaux 1994; Tingay et al. 1997; Kumlehn et al. 2006). A further alternative which has been explored is the *E. coli* gene *manA*, which encodes an enzyme driving the conversion of mannose 6-phosphate to fructose 6-phosphate; the principle was that selection could be imposed by providing mannose as the sole source of organic carbon in the culture medium (Reed et al. 2001). However, the method proved to be less effective than had been hoped for, as media having a high mannose content were toxic to the explants. As a result, to retain viability, it became necessary to include conventionally used sugars alongside with some mannose. Most current barley transformation methods utilize as the selectable marker the *E. coli HPT* gene, because hygromycin proved to be highly effective, so that the frequency of non-transgenic escapes is comparatively low. In approaches where a second selectable marker is required, for example, when an already transgenic barley plant needs to be retransformed with a different gene, the *BAR* gene is the usual choice (Gurushidze et al. 2014).

Once the selectable marker gene has performed its function of facilitating the generation of transgenic plants, it becomes redundant. Although the marker genes in current use for plants are not known to present any health risk or danger to the environment, European regulations imposed on crop plant genetic engineering require that transgenic crops newly approved for commercial production must be free of marker genes. The removal of markers is helpful too where sequential transformation is envisaged, as it allows for the same marker gene to be used

repeatedly. Several strategies have been pursued to generate selectable marker-free transgenic lines. Matthews et al. (2001) created binary transformation vectors carrying the effector transgene and the marker gene each within its own T-DNA, with the two T-DNAs being separated from one another by a spacer sequence. While two thirds of the resulting primary transgenics carried both T-DNAs, a significant proportion inherited the effector transgene independent of the marker gene. The separation of target and marker gene is simplified by the inclusion of doubled haploid technology, which allows for the rapid acquisition of marker-free target gene homozygotes (Kapusi et al. 2013). Through the use of isolated ovules for agroinoculation, Holme et al. (2006) avoided the requirement of marker genes altogether; the downside was a much reduced transformation efficiency.

21.2.3 Detection of Transgenesis

PCR provides a routine method for checking for transgenicity. However, firm evidence for the genomic integration of the transgene is essential, especially in the context of agroinoculation, since *A. tumefaciens* (or other contaminating microbes) cells can persist within primary transgenic plants and are even known to be carried into the following sexual generation (Langridge et al. 1992). Tests based on phenotype (e.g. herbicide resistance or accumulation of GUS) can be misleading, since persisting bacteria are able to successfully express the transgene, even though it is typically driven by a viral or a plant promoter. DNA hybridization analysis therefore provides the best evidence for transgene integration. Although the transgene integration site is deemed to be random with respect to both biolistics- and *Agrobacterium*-based transgenic events (Salvo-Garrido et al. 2004) and transgene expression proved to be not as much dependent on the genomic position as formerly thought (provided transgenic plant formation is based upon an active selectable marker) (Jackson et al. 2001), it can be of interest to identify the transgene integration site (Kapusi et al. 2012a). Biolistics-based transfer typically produces an array of configurations involving multiple transgene copies and integration sites, whereas *Agrobacterium*-mediated transformation tends to favour a single integration site; and if more than one T-DNA copy is present at a single site, these are preferentially arranged as tandem arrays (Stahl et al. 2002). Unexpectedly, the binary vector backbone sequence is quite frequently integrated along with the T-DNA in transgenic barley produced using *Agrobacterium* (Lange et al. 2006). Ideally, transgenesis would target a single cell, which, following its proliferation and regeneration, produces a homogeneously transgenic plant. However, in practice, the explant comprises many cells, only a small proportion of which have a transgene integrated in their nucleus. Non-transgenic cells in close proximity to transformed ones can survive the selection step thanks to local detoxification of the selective agent, and these could end up as a founder of a cell line within a regenerating plant. For this reason, it is commonplace to find genetic mosaicism in primary transgenics, manifested as a lower than expected ratio of transgene-

containing segregants in the T1 generation (Kumlehn et al. 2006). It is also conceivable for a putatively transgenic regenerant to be mosaic for more than one independent transgenic event. As a consequence of the common mosaicism among primary transgenic plants, the phenotypic assessment of transgenesis needs to be delayed to the subsequent generations, where any chimerism is sieved out by meiosis. A comprehensive analysis of transgenicity requires the demonstration that the transgene is successfully transcribed (typically using either RNA gel blotting, RT-PCR or in situ hybridization) and translated (e.g. via visual detection of reporter gene product or an immunoassay).

21.3 Genetic Manipulation

21.3.1 Regulation of Transgene Expression and the Accumulation of Recombinant Transgene Product

The effect of the presence of a transgene on phenotype depends heavily on how the gene is regulated. Most transgene constructs incorporate a constitutive promoter; in barley, functionally well-proven ones are maize *UBIQUITIN-1*, rice *ACTIN1* and the cauliflower mosaic virus (CaMV) *35S* sequences (Furtado and Henry 2005; Vickers et al. 2006). The activity of such so-called constitutive promoters nevertheless can vary substantially from cell type to cell type, as well as in response to the environment (Custers et al. 1999). A comprehensive coverage of the choices of available promoters for the Triticeae cereals has been given by Hensel et al. (2011). Promoter sequences obtained from a Poaceae species are usually effective in barley, while those derived from a dicotyledonous species need always be pre-validated experimentally by an analysis of their effectiveness in driving a reporter gene, since the risk of functional incompatibility is relatively high. In recent years, it has been realized that transgene expression can be enhanced by the inclusion of an intron within the transgene coding sequence, as demonstrated in barley by Bartlett et al. (2009). The enhancer sequence associated with the CaMV *35S* promoter is capable of raising the transcription level of genes in its vicinity (over a range of several kb of sequence) irrespective of the genes' relative orientation one to another (Yoo et al. 2005). The specificity of a promoter can however be compromised through this enhancing effect (Zheng et al. 2007). Own unpublished data have shown that the use of a doubled enhanced CaMV *35S* promoter to drive a selectable marker simultaneously enhanced the expression of an adjacent *GFP* transgene driven by a different constitutive promoter. Despite a significant research effort, as yet there is no reliable means of inducing transgene expression in barley using a chemical trigger. However, the recent successful use of an estradiol-inducible promoter in the model grass *Brachypodium distachyon* offers the prospect of soon being in a position to facilely regulate transgene activity in barley as well (Valdivia et al. 2013).

In addition to regulating the timing and location of transgene expression, it is possible to control the intracellular site of accumulation of the transgene product. Targeting the export of the protein to a specific cellular compartment is typically achieved by the addition to the transgene coding sequence of the relevant signal peptide. A recent example of this intervention has been described by Daghma et al. (2014), who coupled *GFP* to the SV40 virus nuclear localization signal, a measure which helped to analyse the developmental patterns occurring in barley embryogenic pollen culture via nucleus-specific fluorescence (see also Chap. 20).

The strict (but unusual) definition of over-expression relates to the up-regulation of the transcription of a native gene without affecting either the timing or the location of transcription: this can be achieved by increasing gene dosage using a genomic construct including the gene under study along with its native promoter. The same type of construct is also particularly useful for characterizing a loss-of-function mutant via the complementation approach [for examples, see Stein et al. (2005) and Pourkheirandish et al. (2014)]. The most common transgenic strategy used to verify gene function or to alter plant performance makes changes to the timing and/or location of a transgene's native expression by driving it with a heterologous, strong constitutive promoter. More sophisticated approaches to crop improvement are based upon designing a judicious combination between a specific promoter and the transgene. An illustrative recent example in barley has been provided by Seiler et al. (2014) in their coupling of the drought-induced *HvLEA* promoter with genes governing the level of the phytohormone abscisic acid, a strategy which succeeded in improving the crop's level of tolerance to water deficit during grain development. Although genetic engineering is not confined to the expression of genes in heterologous hosts, this approach does hold considerable potential for crop improvement. For example, Risk et al. (2013) were able to show that the expression in barley of the wheat leaf rust resistance gene *Lr34* conferred resistance against both leaf rust and powdery mildew.

Just as transgenesis can be used to up-regulate genes, so it can also be exploited for gene knock-down and knock-out. Co-suppression and antisense technology were the pioneering approaches used for gene silencing; but more recently, double-stranded RNA-forming hairpin constructs have been preferred (Himmelbach et al. 2007), since these have proven to be more efficient and reliable. Downregulation approaches which rely on the production in planta of a gene-specific short double-stranded RNA operate by either driving the degradation of the target mRNA or by inhibiting its translation (Brodersen et al. 2008). The underlying principle of sequence homology between the interfering RNA and its target gene means that it can be focused not only to a single gene but in some cases also to several related target sequences—which can be a disadvantage, as well as an advantage. The more recently established principle of host-induced gene silencing takes advantage of the fact that most eukaryotes possess an endogenous RNA interference machinery. The derived downregulation strategy is based on the host's ability to produce hairpin RNAs which form double-stranded RNAs matching the sequence of a pathogen's gene; when the incoming pathogen is taking up these molecules, its own target gene is downregulated, thereby weakening its

capacity to damage the host. Nowara et al. (2010) demonstrated that the expression in barley of a hairpin construct specific to the powdery mildew pathogenic fungus *Avra10* gene resulted in a reduced level of fungal development. This principle is seen as a potentially highly effective and safe means of crop protection, as it operates solely at the RNA level, avoiding the formation of any transgene-derived protein or modified metabolite. A second example sought to render barley immune to the highly damaging fungus causing head blight (*Fusarium graminearum*) by expressing a hairpin RNA specific to a group of fungal sterol demethylase genes known to be essential for pathogenicity (Koch et al. 2014).

21.3.2 Microbial Recombination Systems

The long established adenoviral CRE-*lox* and *Saccharomyces* FLP-*FRT* recombination systems have been transferred to a number of plant species (including the cereals) and used as a means to excise selectable marker genes, to activate genes via the restoration of sequence functionality or to remove redundant transgene copies [for a review, see Wang et al. (2011)]. In barley, the *Streptomyces* phage phiC31 integrase has been shown able to perform irreversible, genomic site-specific DNA excision (Kapusi et al. 2012b). In brief, transgenic plants expressing the gene encoding phiC31 integrase were crossed with a transgenic line carrying *GFP* flanked by the phiC31 integrase *attB* and *attP* recognition sites, with the *GFP* itself positioned between a constitutive promoter and a downstream *GUS* coding sequence. Recombinase-mediated excision of the *GFP* sequence not only resulted in the loss of *GFP* expression but also activated *GUS*, which unambiguously indicated that recombination had taken place.

21.3.3 Chromosome Engineering

Both transgene stacking (e.g. for pathway engineering) and the transfer of transgenes between different host cultivars without disturbing the genetic background would benefit from the development of accessory mini-chromosomes able to both harbour multiple transgenes on genomic landing pads and replicate through mitosis and meiosis. The first step in barley towards the construction of mini-chromosomes was achieved by truncating endogenous chromosomes via the *Agrobacterium*-mediated interstitial integration of conserved telomeric sequences, which themselves functionally define chromosome ends (Kapusi et al. 2012a). The formation of novel telomeres, including the 'seeding' of new telomere repeats and the generative transmission of the truncated chromosomes, could be unequivocally demonstrated. However, as yet, the truncations were mostly concentrated close to the original chromosome ends, and their inheritance was not as regular as had been hoped.

21.3.4 Genome Engineering

Genome engineering, in which any genomic sequence can be modified without the prior integration of heterologous recombination sites, is set to revolutionize both the technology surrounding the functional validation of genes and applied biotechnology practice. A key feature is the induction of a double-stranded break in any target locus of choice. Various platforms are under development, including those relying on zinc-finger nucleases (ZFNs; Bibikova et al. 2003; Shukla et al. 2009), on transcription activator-like effector nucleases (TALENs; Bogdanove et al. 2010; Li et al. 2012) and on RNA-guided nucleases (Jinek et al. 2012; Li et al. 2013; Shan et al. 2013). As yet, only two reports describing targeted genome modification in barley have been published, both involving the use of TALENs (Wendt et al. 2013; Gurushidze et al. 2014); these proteins feature a customizable DNA-binding domain and a *FokI* endonucleolytic domain and are able to specifically cleave in planta virtually any genomic DNA sequence of interest. The process induces targeted genetic modifications following the intervention of the host cells' endogenous DNA repair machinery. As the DNA-binding domain of TAL effectors consists of numerous tandem repeats each recognizing one specific nucleotide of the DNA target motif (Boch et al. 2009; Moscou and Bogdanove 2009), the customization of TALENs is at present a challenging procedure. Still, Golden Gate cloning already provides a reliable platform for the modular assembly of TALEN-encoding sequences (Weber et al. 2011; Cermak et al. 2011). Pairs of TALEN genes can be either transiently or stably expressed in host tissue. Wendt et al. (2013) have shown that TALEN-induced double-stranded breaks in the promoter of the barley *PHYTASE* gene (*HvPAPhy_a*) induced a variety of localized deletions, with, on average, 25 % of antibiotic-resistant primary transgenics being mutated at the target site. The same study also revealed TALEN-induced mosaicism involving as many as nine sequence variants of the targeted promoter region in a single barley transgenic plant, indicating that mutations must have occurred independently in different cells. However, a subsequent investigation established that none of these mutations could be recovered in the mutants' selfed progeny (Wendt et al. 2014). The current assumption is that the CaMV *35S* promoter used to drive TALEN expression was too weak during early embryogenesis to induce mutations in germ line founder cells. Gurushidze et al. (2014), however, have successfully produced targeted knock-outs in barley using TALENs. Here, a *GFP*-specific TALEN pair was designed and expressed in embryogenic pollen cultures of a *GFP*-carrying line. More than 20 % of the primary transgenics carried a mutated *GFP* sequence, and loss-of-function mutations could be ascribed to deletions of 4–36 nucleotides of the target motif. The altered *GFP* alleles were faithfully transmitted through meiosis, and no segregation was noted among the progeny of two independent mutants, which indicated instant homozygosity of the mutations and no chimerism in the primary transgenic plants.

Conclusions and Perspectives

Although genetic transformation of barley has become a well-established, relatively efficient and reproducible procedure, it is in routine operation in only a small number of laboratories worldwide and is very dependent on trained and skilled personnel. While cv. 'Golden Promise' is readily transformable, there is a growing list of cultivars which are less amenable to transformation, but for which efficient protocols would be highly desirable—this includes current elite breeders' lines, cv. 'Bowman' (the genetic background of many phenotypically well-described mutants) and cvs. 'Optic', 'Sebastian', 'Barke' and 'Morex' (for which large TILLING populations have been generated); the latter two cultivars are also associated with a comprehensive range of genomic data (see also Chaps. 14 and 15).

In addition to targeted knock-outs, as alluded to in a previous section, increasingly sophisticated designer nuclease technology-based strategies are likely to be established soon to generate targeted sequence insertions and exactly predictable genetic modifications. Both of these manipulations rely on homologous recombination to repair double-stranded breaks and require a DNA repair template which contains sequences homologous to the flanking regions of the genomic site to be edited. In plants, this technology is still largely confined to the cellular level, but once established in whole plants, it is likely to kick-start a new, even more precise generation of genetic engineering.

Although there are, as yet, no transgenic barley cultivars approved for commercial cultivation, a growing number of transgenic lines expressing enhanced traits continue to be developed, and the technical framework required for breeding genetically engineered (GE) barley is at a stage where it could soon become a commercially viable undertaking. Still complicating the picture is the heavy regulatory framework surrounding the release of GE crops and the legal complexities associated with ownership of intellectual property over techniques and genes. These issues will need to be at least partially resolved before the promise of transgenic barley can be translated into the release of cultivars providing measurable benefit to both the barley producer and consumer.

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References

Bartlett JG, Alves SC, Smedley M, Snape JW, Harwood WA (2008) High-throughput Agrobacterium-mediated barley transformation. Plant Methods 4:22

Bartlett JG, Snape JW, Harwood WA (2009) Intron-mediated enhancement as a method for increasing transgene expression levels in barley. Plant Biotechnol J 7:856–866

Bibikova M, Beumer K, Trautman JK, Carroll D (2003) Enhancing gene targeting with designed zinc finger nucleases. Science 300:764

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U (2009) Breaking the code of DNA binding specificity of TAL-Type III effectors. Science 326:1509–1512

Bogdanove AJ, Schornack S, Lahaye T (2010) TAL effectors: finding plant genes for disease and defense. Curr Opin Plant Biol 13:394–401

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O (2008) Widespread translational inhibition by plant miRNAs and siRNAs. Science 320:1185–1190

Carlson AR, Letarte J, Chen J, Kasha KJ (2001) Visual screening of microspore-derived transgenic barley (*Hordeum vulgare* L.) with green fluorescent protein. Plant Cell Rep 20:331–337

Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39:e82

Cho M-J, Wong JH, Marx C, Jiang W, Lemaux PG, Buchanan BB (1999) Overexpression of thioredoxin *h* leads to enhanced activity of starch debranching enzyme (pullulanase) in barley grain. Proc Natl Acad Sci U S A 96:14641–14646

Choi I-R, Stenger DC, Morris TJ, French R (2000) A plant virus vector for systemic expression of foreign genes in cereals. Plant J 23:547–555

Custers JBM, Snepvangers SCHJ, Jansen HJ, Zhang L, van Lookeren Campagne MM (1999) The 35S-CaMV promoter is silent during early embryogenesis but activated during nonembryogenic sporophytic development in microspore culture. Protoplasma 208:257–264

Daghma DES, Hensel G, Rutten T, Melzer M, Kumlehn J (2014) Cellular dynamics during early barley pollen embryogenesis revealed by time-lapse imaging (submitted)

Delventhal R, Zellerhoff N, Schaffrath U (2011) Barley stripe mosaic virus-induced gene silencing (BSMV-IGS) as a tool for functional analysis of barley genes potentially involved in nonhost resistance. Plant Signal Behav 6:867–869

Ding XS, Schneider WL, Chaluvadi SR, Mian MAR, Nelson RS (2006) Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Mol Plant Microbe Interact 19:1229–1239

Douchkov D, Nowara D, Zierold U, Schweizer P (2005) A high-throughput gene silencing system for the functional assessment of defense-related genes in barley epidermal cells. Mol Plant Microbe Interact 18:755–761

French R, Janda M, Ahlquist P (1986) Bacterial gene inserted in an engineered RNA virus: efficient expression in monocotyledonous plant cells. Science 231:1294–1297

Funatsuki H, Kuroda H, Kihara M, Lazzeri PA, Mueller E, Lörz H, Kishinami I (1995) Fertile transgenic barley generated by direct DNA transfer to protoplasts. Theor Appl Genet 91:707–712

Furtado A, Henry RJ (2005) The wheat Em promoter drives reporter gene expression in embryo and aleurone tissue of transgenic barley and rice. Plant Biotechnol J 3:421–434

Goedeke S, Hensel G, Kapusi E, Gahrtz M, Kumlehn J (2007) Transgenic barley in fundamental research and biotechnology. Transgenic Plant J 1:104–117

Gurushidze M, Hensel G, Hiekel S, Schedel S, Valkov V, Kumlehn J (2014) True-breeding targeted gene knock-out in barley using designer TALE-nuclease in haploid cells. PLoS One 9:e92046

Hensel G, Valkov V, Middlefell-Williams J, Kumlehn J (2008) Efficient generation of transgenic barley: the way forward to modulate plant-microbe interactions. J Plant Physiol 165:71–82
Hensel G, Kastner C, Oleszczuk S, Riechen J, Kumlehn J (2009) Agrobacterium-mediated gene transfer to cereal crop plants: current protocols for barley, wheat, triticale and maize. Int J Plant Genomics, Article ID 835608, 9 pp
Hensel G, Himmelbach A, Chen W, Douchkov DK, Kumlehn J (2011) Transgene expression systems in the Triticeae cereals. J Plant Physiol 168:30–44
Himmelbach A, Zierold U, Hensel G, Riechen J, Douchkov D, Schweizer P, Kumlehn J (2007) A set of modular binary vectors for the transformation of cereals. Plant Physiol 145:1192–1200
Holm PB, Olsen O, Schnorf M, Brinch-Pedersen H, Knudsen S (2000) Transformation of barley by microinjection into isolated zygote protoplasts. Transgenic Res 9:21–32
Holme IB, Brinch-Pedersen H, Lange M, Holm PB (2006) Transformation of barley (Hordeum vulgare L.) by Agrobacterium tumefaciens infection of in vitro cultured ovules. Plant Cell Rep 25:1325–1335
Holme IB, Brinch-Pedersen H, Lange M, Holm PB (2008) Transformation of different barley (Hordeum vulgare L.) cultivars by Agrobacterium tumefaciens infection of in vitro cultured ovules. Plant Cell Rep 27:1833–1840
Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant J 30:315–327
Horvath H, Huang J, Wong O, Kohl E, Okita T, Kannangara CG, von Wettstein D (2000) The production of recombinant proteins in transgenic barley grains. Proc Natl Acad Sci U S A 97:1914–1919
Ihlow A, Schweizer P, Seiffert U (2008) A high-throughput screening system for barley/powdery mildew interactions based on automated analysis of light micrographs. BMC Plant Biol 2008:8
Jackson SA, Zhang P, Chen WP, Phillips RL, Friebe B, Muthukrishnan S, Gill BS (2001) High-resolution structural analysis of biolistic transgene integration into the genome of wheat. Theor Appl Genet 103:56–62
Jähne A, Becker D, Brettschneider H, Lörz H (1994) Regeneration of transgenic, microspore-derived, fertile barley. Theor Appl Genet 89:525–533
Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821
Kapusi E, Ma L, Teo CH, Hensel G, Himmelbach A, Schubert I, Mette MF, Kumlehn J, Houben A (2012a) Telomere-mediated truncation of barley chromosomes. Chromosoma 121:181–190
Kapusi E, Kempe K, Rubtsova M, Kumlehn J, Gils M (2012b) PhiC31 integrase-mediated site-specific recombination in barley. PLoS One 7:e45353
Kapusi E, Hensel G, Coronado M-J, Broeders S, Marthe C, Otto I, Kumlehn J (2013) The elimination of a selectable marker gene in the doubled haploid progeny of co-transformed barley plants. Plant Mol Biol 81:149–160
Koch A, Kumar N, Weber L, Keller H, Imani J, Kogel KH (2014) Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase–encoding genes confers strong resistance to Fusarium species. Proc Natl Acad Sci U S A 110:19324–19329
Kumlehn J, Serazetdinova L, Hensel G, Becker D, Lörz H (2006) Genetic transformation of barley (Hordeum vulgare L.) via infection of androgenetic pollen cultures with Agrobacterium tumefaciens. Plant Biotechnol J 4:251–261
Kumlehn J, Zimmermann G, Berger C, Marthe C, Hensel G (2010) Triticeae cereals. In: Kempken F, Jung C (eds) Biotechnology in agriculture and forestry, vol 64, Genetic modification of plants. Springer, Berlin, pp 287–306
Lange M, Vincze E, Moller MG, Holm PB (2006) Molecular analysis of transgene and vector backbone integration into the barley genome following Agrobacterium-mediated transformation. Plant Cell Rep 25:815–820
Langridge P, Brettschneider R, Lazzeri P, Lörz H (1992) Transformation of cereals via Agrobacterium and the pollen pathway: a critical assessment. Plant J 2:631–638

Lazzeri PA, Brettschneider R, Lührs R, Lörz H (1991) Stable transformation of barley via PEG-induced direct DNA uptake into protoplasts. Theor Appl Genet 81:437–444

Li T, Liu B, Spalding MH, Weeks DP, Yang B (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat Biotechnol 30:390–392

Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. Nat Biotechnol 31:688–691

Marzin S, Mihaly R, Pauk J, Schweizer P (2008) A transient assay system for the assessment of cell-autonomous gene function in dehydration-stressed barley. J Exp Bot 59:3359–3369

Matthews PR, Wang MB, Waterhouse PM, Thornton S, Fieg S, Gubler F, Jacobsen JV (2001) Marker gene elimination from transgenic barley, using co-transformation with adjacent 'twin T-DNAs' on a standard Agrobacterium transformation vector. Mol Breed 7:195–202

Meng Y, Moscou MJ, Wise RP (2009) Blufensin1 negatively impacts basal defense in response to barley powdery mildew. Plant Physiol 149:271–285

Moscou MJ, Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. Science 326:1501

Murray F, Brettell R, Matthews P, Bishop D, Jacobsen J (2004) Comparison of Agrobacterium-mediated transformation of four barley cultivars using the GFP and GUS reporter genes. Plant Cell Rep 22:397–402

Nobre J, Davey MR, Lazzeri PA, Cannell ME (2000) Transformation of barley scutellum protoplasts: regeneration of fertile transgenic plants. Plant Cell Rep 19:1000–1005

Nowara D, Gay A, Lacomme C, Shaw J, Douchkov D, Hensel G, Kumlehn J, Schweizer P (2010) Host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis affects its development. Plant Cell 22:3130–3141

Oikawa A, Rahman A, Yamashita T, Taira H, Kidou S (2007) Virus-induced gene silencing of P23k in barley leaf reveals morphological changes involved in secondary wall formation. J Exp Bot 58:2617–2625

Pourkheirandish M, Hensel G, Kilian B, Willcox G, Senthil N, Chen G, Sameri M, Sakuma S, Dhanagond S, Sharma R, Gottwald S, Azhaguvel P, Nair S, Tagiri A, Yukuhiro F, Nagamura Y, Kanamori H, Matsumoto T, Middleton C, Wicker T, Walther A, Stein N, Kumlehn J, Sato K, Komatsuda T (2014) Anthropogenic selection of independent deletions in NON-BRITTLE RACHIS 1 and NON-BRITTLE RACHIS 2 were responsible for the domestication of wild barley (submitted)

Radchuk V, Borisjuk L, Radchuk R, Steinbiss HH, Rolletschek H, Broeders S, Wobus U (2006) Jekyll encodes a novel protein involved in the sexual reproduction of barley. Plant Cell 18:1652–1666

Reed J, Privalle L, Powell ML, Meghji M, Dawson J, Dunder E, Suttie J, Wenck A, Launis K, Kramer C, Chang YF, Hansen G, Wright M (2001) Phosphomannose isomerase: an efficient selectable marker for plant transformation. In Vitro Cell Dev Biol Plant 37:127–132

Risk JM, Selter LL, Chauhan H, Krattinger SG, Kumlehn J, Hensel G, Viccars LA, Richardson TM, Buesing G, Troller A, Lagudah ES, Keller B (2013) The wheat Lr34 gene provides resistance against multiple fungal pathogens in barley. Plant Biotechnol J 11:847–854

Salmenkallio-Marttila M, Aspegren K, Akerman S, Kurten U, Mannonen L, Ritala A, Teeri TH, Kauppinen J (1995) Transgenic barley (*Hordeum vulgare* L.) by electroporation of protoplasts. Plant Cell Rep 15:301–304

Salvo-Garrido H, Travella S, Bilham LJ, Harwood WA, Snape JW (2004) The distribution of transgene insertion sites in barley determined by physical and genetic mapping. Genetics 167:131–1379

Seiler C, Harshavardhan VT, Reddy PS, Hensel G, Kumlehn J, Selvaraj GL, Rajesh K, Korzun V, Wobus U, Lee J, Selvaraj G, Sreenivasulu N (2014) Abscisic acid flux alterations result in differential ABA signalling responses and impact assimilation efficiency in barley under terminal drought stress. Plant Physiol 164:1677–1696

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C (2013) Targeted genome modification of crop plants using a CRISPR–Cas system. Nat Biotechnol 31:686–688

Shim YS, Pauls KP, Kasha KJ (2009) Transformation of isolated barley (Hordeum vulgare L.) microspores. I. The influence of pretreatments and osmotic treatment on the time of DNA synthesis. Genome 52:166–174

Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459:437–441

Stahl R, Horvath H, Van Fleet J, Voetz M, von Wettstein D, Wolf N (2002) T-DNA integration into the barley genome from single and double cassette vectors. Proc Natl Acad Sci U S A 99:2146–2151

Stein N, Perovic D, Kumlehn J, Pellic B, Stracke S, Streng S, Ordon F, Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive bymovirus resistance in Hordeum vulgare L. Plant J 42:912–922

Stöldt A, Wang X-H, Lörz H (1996) Primary callus as source of totipotent barley (Hordeum vulgare L.) protoplasts. Plant Cell Rep 16:137–141

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) Agrobacterium tumefaciens-mediated barley transformation. Plant J 11:1369–1376

Valdivia ER, Herrera MT, Gianzo C, Fidalgo J, Revilla G, Zarra I, Sampedro J (2013) Regulation of secondary wall synthesis and cell death by NAC transcription factors in the monocot Brachypodium distachyon. J Exp Bot 64:1333–1343

Vickers CE, Xue G, Gresshoff PM (2006) A novel cis-acting element, ESP, contributes to high-level endosperm-specific expression in an oat globulin promoter. Plant Mol Biol 62:195–214

Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol 104:37–48

Wang Y, Yau YY, Perkins-Balding D, Thomson JG (2011) Recombinase technology: applications and possibilities. Plant Cell Rep 30:267–285

Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S (2011) A modular cloning system for standardized assembly of multigene constructs. PLoS One 6:e16765

Wendt T, Holm PB, Starker CG, Christian M, Voytas DF, Brinch-Pedersen H, Holme IB (2013) TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. Plant Mol Biol 83:279–285

Wendt T, Holm PB, Starker CG, Christian M, Voytas DF, Brinch-Pedersen H, Holme IB (2014) TALEN-mediated mutagenesis in primary barley transformants. Proceedings on plant transformation technologies III, Vienna, 12–15 Feb 2014, p 29

Williams-Carrier RE, Lie YS, Hake S, Lemaux PG (1997) Ectopic expression of the maize kn1 gene phenocopies the Hooded mutant of barley. Development 124:3737–3745

Yeo FKS, Hensel G, Vozábová T, Martin-Sanz A, Marcel TC, Kumlehn J, Niks RE (2014) Golden SusPtrit – a genetically well transformable barley line for studies on the resistance to rust fungi. Theor Appl Genet 127:325–337

Yoo SY, Bomblies K, Yoo SK, Yang JW, Choi MS, Lee JS, Weigel D, Ahn JH (2005) The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. Plant Cell Rep 221:523–530

Zhang S, Cho M-J, Koprek T, Yun R, Bregitzer P, Lemaux PG (1999) Genetic transformation of commercial cultivars of oat (Avena sativa L.) and barley (Hordeum vulgare L.) using in vitro shoot meristematic cultures derived from germinated seedlings. Plant Cell Rep 18:959–966

Zheng X, Deng W, Luo K, Duan H, Chen Y, McAvoy R, Song S, Pei Y, Li Y (2007) The cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters. Plant Cell Rep 26:1195–1203

Zimmermann G, Bäumlein H, Mock HP, Himmelbach A, Schweizer P (2006) The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. Plant Physiol 142:181–192

Chapter 22
Whole-Plant Phenomics

Mark L. Crowe, Timothy J. March, and Joanne Tilbrook

22.1 Introduction

Phenotyping, the measuring of an organism's observable characteristics, is perhaps one of the oldest techniques in plant science. Since the advent of agriculture some 10,000 years ago, farmers have assessed plants on their physical attributes, selecting for and propagating those that best suited their particular needs. While the selection itself may often have been inadvertent, it is clear that selection by phenotyping was a key to crop improvement and agricultural progress.

Breeding science and our understanding of genetics have come a long way since, but phenotyping remains an important tool among other advanced technologies in regular use in breeding programmes; after all, the ultimate goal of any breeding programme is a specific improved phenotype. High-quality phenotyping is an essential element in understanding how the genetics of a plant leads to a particular phenotype.

Conventionally, most plant and animal phenotyping has focused on detailed measurement of a relatively small number of traits that are biologically relevant,

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such as plant height, biomass or leaf area, or of direct production importance, such as grain yield and drought tolerance. The techniques used in these conventional approaches are often time consuming and frequently destructive; this limits both the numbers of plants that are feasible to study in a given experiment and the number of time points that can be sampled for dynamic phenotypes. This lack of scale significantly restricts the power of many experiments, particularly productivity traits which are typically complex and have low heritability (e.g. see Ceccarelli and Grando 1996; Blum 2004).

To address some of these shortcomings, inspired in part by the data generation-focused techniques of genomics research, phenotyping has adopted an analogous approach with the development of 'phenomics'. The field of whole-plant phenomics combines robotics, image capture and high-performance computational analysis and provides plant scientists with the ability to characterise dozens of phenotypes on thousands of plants daily. Phenomics platforms are also often designed for experimentation directly on crop plants, including barley; this capability is driving technical developments in laboratory-based studies directly on commercially important species, reducing both the need for model species experimentation and the challenges of field trials.

The throughput and accuracy offered by plant phenomics systems provide plant physiologists and geneticists with the opportunity to study large mapping populations, perform detailed associations studies or screen many more plants for rare specific phenotypic characteristics than would otherwise be possible. Combined with the ability to record data in multiple visible and non-visible wavelengths and to study root structure or entire field trial sites, these techniques are generating previously unachievable insights into many aspects of plant biology and productivity.

Already, phenomics research in barley has produced novel data about response and tolerance to drought and salinity stress, and applications are being developed that will enhance our understanding of growth patterns, water and nutrient usage and photosynthetic efficiency, among many other traits. At the same time, phenomics techniques are being applied in large-scale genetic screening and breeding systems. These advances will provide plant physiologists and geneticists with the opportunity to benefit from the ever-increasing genomics resources that are available in barley and other crop species.

22.2 Benefits of the Phenomics Approach

22.2.1 Large-Scale, Non-destructive, Multiple Phenotypes

Conventional plant phenotyping projects are typically small scale, focus on a limited number of measurements and involve manual, time-consuming and often destructive testing. Using phenomics, more phenotypes on larger numbers of plants can be studied, greatly improving the statistical power of an experiment to detect

subtle differences within and between populations. The non-destructive nature of phenomics phenotyping also enables repeated measures on the same plants, which are statistically more powerful than destructive measurements on multiple plants at multiple time points because of the reduction of unrelated biological variation in the dataset.

22.2.2 Accuracy and Reproducibility

Phenotyping in traditional cereal crop breeding programmes may include tens of thousands of individual plots, all of which are visually inspected by a breeder and other field workers. Some phenotyping consists of direct measurements, but a significant portion is the result of a qualitative assessment being translated into a score, for example, the use of early vigour as an estimation of future growth rate or stress tolerance as described by Montes et al. (2011). Unfortunately, in addition to the inherent variability in field trials from soil, site and weather variation, visual assessment is prone to inconsistency and potential, if unconscious, bias.

This subjectivity can be reduced by the use of phenomics techniques, resulting in superior phenotyping results. Automated phenomics systems have proven to be superior to expert assessment or other nonautomated measurement for both qualitative and quantitative traits, such as colour and leaf area (Van Tunen 2011; Arvidsson et al. 2011).

22.2.3 Non-visible Characteristics

Phenomics is not limited to traits that can be observed in visible light, and a wide range of imaging technologies can be used to collect information about a plant. Infrared, ultraviolet (fluorescence), nuclear magnetic resonance (NMR or MRI), X-ray and terahertz (THz) imaging can all be used to detect features of the plant invisible to the human eye (Sirault et al. 2009; Jansen et al. 2009; Scholes and Rolfe 2009; Segal et al. 2008; Tracy et al. 2011; Chan et al. 2007). These are useful not only for studying internal or subsoil plant architecture using NMR and X-ray computed tomography (CT) but also allow the measurement of specific physiological features which have characteristic spectral patterns in non-visible wavelengths; perhaps the most common example of the latter is the use of infrared thermography to measure plant temperature and thus infer transpiration activity, stomatal conductance and water stress (Munns et al. 2010; Maes and Steppe 2012).

22.2.4 Forward and Reverse Genetics

High-throughput genetic screens are a powerful way to discover genes associated with specific plant phenotypes or, conversely, to characterise phenotypes affected by particular genes (Furbank and Tester 2011). These screens can be performed with populations produced using random mutagenesis or targeted transformation with large libraries of gene constructs or can be in the form of association studies using genetically diverse populations such as those generated using Multi-parent Advanced Generation Inter-Cross (MAGIC) (Cavanagh et al. 2008). Phenotyping such populations can be largely automated using phenomics platforms. For example, the TraitMill platform developed by CropDesign has the capacity to screen more than 140,000 transformed plants per year using image-based phenotyping (http://www.cropdesign.com/tech_traitmill.php).

22.2.5 Trait Dissection

Key productivity traits such as grain yield are extremely complex as they are influenced by many underlying sub-traits such as height, leaf area and tiller number. This can result in low trait heritability, particularly when measuring yield across different growing conditions (Furbank and Tester 2011). Through the simultaneous measurement of multiple traits, repeated measurements over time and the use of accurately controlled environmental conditions, plant phenomics provides a way to dissect complex traits into more heritable sub-traits (Roy et al. 2011). Measurement can be automated and done in real time, and multivariate statistical analysis used to identify sub-traits significantly contributing to overall plant performance in different environments. The potential of trait dissection using phenomics approaches was demonstrated by Rajendran et al. (2009), in that instance, in the study of components of salinity tolerance.

22.2.6 Limitations

Despite the many benefits of the phenomics approach to plant phenotyping, there are limitations on what can be achieved. Chief among these are the restrictions on high-throughput phenotyping of roots in soil. A number of options for studying roots are described in Sect. 4.3, but all are currently restricted in terms of cost and throughput (Zhu et al. 2011).

To achieve the high throughput, accuracy and reproducibility that give phenomics much of its potential and to minimise confounding environmental interactions, most systems are established in controlled and artificial environments. This may limit the aspects of plant physiology that can be studied: for example, no

currently available controlled environment phenomics platform provides the capacity to grow cereal crops in a dense canopy-like arrangement, nor is it feasible to provide pots that would allow the depth of root penetration achieved in the field. While this unnaturalness must be properly considered when planning and undertaking phenomics experiments, it is by no means a critical failure in the technology.

Whole-plant phenotyping is also not currently suited to providing direct information about the essential commercial characteristics of barley plants—yield, grain quality and malting quality. Rather, its strength lies in the potential to improve overall survival and productivity traits such as growth rate and drought tolerance (Berger et al. 2010). However, as technological improvements continue in cameras and software and researchers build knowledge into the relationship between particular spectral patterns and key value traits, this shortcoming is likely to be partially addressed in the future.

Finally, it is important to appreciate that the costs involved in establishing a plant phenomics facility are considerable, often extending into millions of dollars. Consequently, most researchers will require access to a core facility or other third-party provider to perform phenomics experiments. With several publicly available facilities already operating around the world and others in construction, such access is becoming much less of a barrier; indeed, this limitation may even be outweighed by the benefits of expertise and capability gained from using such service providers.

22.3 Technological Approaches

22.3.1 Background

Although this chapter is entitled 'Whole-Plant Phenomics', in practice, few researchers or techniques truly focus on whole plants. More typically, they will study the phenotype of either the above-ground (shoot) or below-ground (root) portions of the plant, with the former in some cases being whole field populations. Because the needs and challenges of these three areas are so different, they are addressed in separate sections below (with the relevant applications in each area summarised in Table 22.1). What unites all three in phenomics is that the approaches are largely imaging based.

Imaging provides an indirect measurement of most phenotypes, with plant height being a rare exception. However, it is the most convenient way to study large numbers of plants in toto; it is faster than other techniques and allows a much greater range of phenotypes to be recorded than would otherwise be possible. Also, whole-plant phenotyping is used to investigate either relative differences or patterns of phenotyping variation, and this information is better captured by imaging than other techniques.

Table 22.1 Summary of applicability of the different phenomics imaging technologies

Imaging technology	Maturity of technology[a]	Above ground	Below ground	Field phenotyping
Visible light; morphometric characteristics, colour analysis	+++	✔	X	✔
Infrared thermography; plant temperature	++	✔	X	✔
Fluorescence; photosynthetic activity	+	✔	X	✔
Terahertz imaging; water distribution	−−	✔	?[b]	X
X-ray (including CT)	−	Possible, but of limited use	✔	X
NMR	−	✔	✔	X
Hyperspectral imaging	+	✔	X	✔

[a]With respect to plant phenotyping applications
[b]It remains unclear whether terahertz imaging can successfully be used for below ground imaging

A major benefit of image-based phenotyping is that, because it is non-destructive with generally minimal effects on the plant, most phenomics systems allow repeated measurements over time. This allows the study of time-dependent phenotypes and developmental patterns without the challenges of the increased technical replication required with destructive testing.

22.3.2 Above-Ground Phenotyping Approaches

22.3.2.1 Visible Light Imaging

Visible light imaging using conventional digital cameras is the simplest and most widely used technique in whole-plant phenotyping. It automates the recording of morphometric data about plant shoots including height, leaf number and branch angles and can provide estimates of total shoot mass and leaf area (Golzarian et al. 2011; Fig. 22.1). Aspects of overall plant architecture including erectness, branching patterns and canopy density can also be identified from visible light imaging.

The value of visible light imaging can be enhanced by combining a series of images taken over time. This allows the gathering of data about growth rate and growth pattern of the plant, which may be for a simple study of overall plant biomass increase or a more detailed investigation into the time of emergence and subsequent growth of specific organs. Time-dependent changes resulting from particular treatments applied during the imaging series, such as drought and salinity tolerance responses in barley, can also be investigated (Harris et al. 2010; Hartmann et al. 2011; Parent 2010).

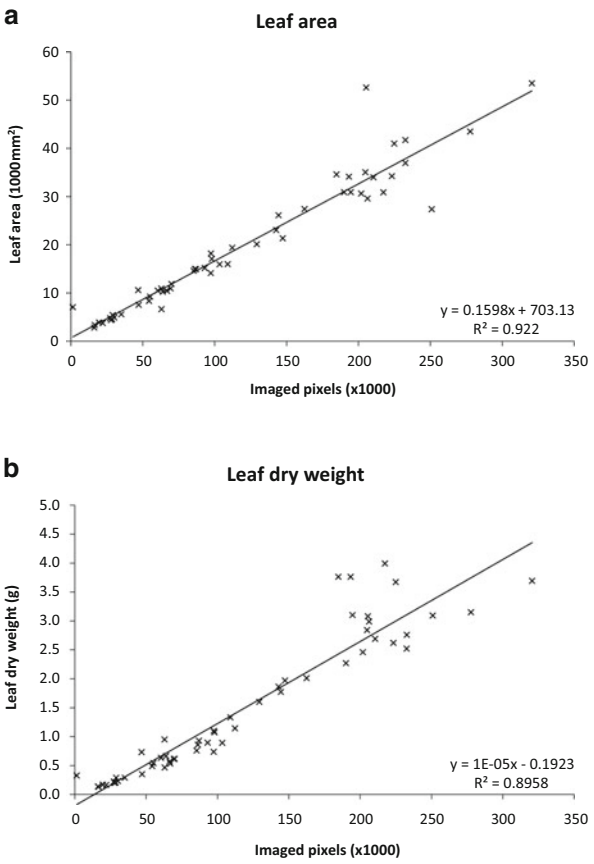

Fig. 22.1 Graphs showing the high linear correlation between imaged area, expressed as the sum of pixels from three images taken at 90° to each other, and actual leaf area and dry weight for wheat plants of up to 6 weeks in age (data courtesy of Bettina Berger, The Plant Accelerator and Boris Parent, Australian Centre for Plant Functional Genomics)

Visible light cameras also provide colorimetric data for use in colour classification algorithms, where individual image pixels are categorised based on their colour balance. This type of analysis can be used to assay features such as leaf senescence (Rajendran et al. 2009), pathogen infection, chlorophyll distribution or any other traits which generate visually detectable variation.

22.3.2.2 Infrared Imaging

Infrared (IR) imaging can be performed using a range of different wavelengths for different purposes. Long-wave, or thermal IR, measures leaf temperature differences. A plant under drought or salinity stress will close stomata and reduce transpiration, which slows photosynthesis and water loss but also reduces the ability of the plant to cool its leaves. Sirault et al. (2009) found that leaf temperature of barley seedlings under salt stress was raised up to 1.6 °C, correlating with reductions in stomatal conductance. Since stomatal conductance is an accurate predictor

of growth rate under stress conditions (Munns et al. 2010), measuring thermal IR can give an instantaneous indication of growth rate under stress.

Certain shorter wavelengths of IR radiation are absorbed by water, and short-wave IR imaging is being developed as a tool to measure water content and distribution within plant tissues (see http://www.plantphenomics.org.au/).

22.3.2.3 Fluorescence Imaging

Illumination of leaves with ultraviolet or short-wave blue light stimulates photosynthetic pigments to fluoresce and can be used to investigate various measures of photosynthetic efficiency (Baker 2008). It has been used in the study of stress-induced changes in photosynthetic capacity; chlorophyll content in leaves of wheat and *Arabidopsis* seedlings varies in response to salt treatment, implying potential applications of fluorescence imaging to estimate salt tolerance (Cuin et al. 2010; Jansen et al. 2009). Leaf chlorophyll fluorescence can also be used to quantify the impacts of plant-pathogen interactions in high-throughput phenomics environments (Scholes and Rolfe 2009).

22.3.2.4 Terahertz Imaging

Terahertz radiation, a wavelength longer than infrared, shows the water status and water distribution of leaves (Wai Lam et al. 2007). The technology is not widely available but has potential for non-destructive data collection.

22.3.2.5 Three-Dimensional Imaging

Most whole-plant phenotyping technologies use systems which produce two-dimensional (2D) images of the plant. Often these are then analysed directly, although some image analysis software does allow the reconstruction of three-dimensional (3D) structures from multiple 2D images. Two-dimensional systems are preferred where there is no specific need for 3D images because they are generally cheaper, simpler and faster to operate than 3D imagers. For larger plants or those with more complex architectures, where overlapping leaves or other structural issues prevent the inter-image mapping required for 3D reconstruction, direct 3D approaches become valuable. Two main methods are being applied in plant phenotyping: LiDAR (light detection and ranging) scanners and TOF (time-of-flight) cameras.

LiDAR uses scanning laser pulses to build up a 3D model. It is relatively slow, taking several minutes per plant, but produces detailed models at millimetre-scale resolution. There is little limitation on target plant size, allowing imaging of mature trees and even entire crop or forest canopies. Indeed, canopy architecture has been one of the principal uses of LiDAR in plant phenomics, although it is now being

increasingly used for single-plant studies (Omasa et al. (2007) and references therein; see also http://www.plantphenomics.org.au/hrppc).

TOF is a relatively recent approach, where the entire image is captured in a single light pulse rather than a point-by-point scan. Consequently image capture speed with TOF cameras is comparable to that of a conventional RGB visible digital camera. The forfeit of this speed is low resolution, with cameras currently only providing around 200×200 pixel images (Klose et al. 2009). This will of course increase over time, with TOF cameras likely to become a valuable technology in whole-plant phenotyping.

22.3.2.6 Hyperspectral Imaging

Many plant pigments and other metabolites produce characteristic reflectance or absorption patterns, and these can potentially be used to identify their relative proportions within the plant. However, the spectral peaks of these compounds may be close or even overlapping; this makes them impossible to distinguish with RGB digital cameras, which detect only three broad and overlapping spectral ranges. Hyperspectral imaging addresses these limitations by recording multiple images in narrow and distinct wavelength ranges.

The most widespread application of hyperspectral imaging in plant science is the use of visible light wavelengths to quantify the major pigment groups of chlorophylls, carotenoids and anthocyanins; this is already being used in commercial plant breeding pipelines (Malone 2011). Short-wave IR hyperspectral imaging offers the potential for detection of water distribution within plant tissues, as well as protein and carbohydrate make-up and content [see reviews in Blackburn (2007); Furbank and Tester (2011)].

22.3.3 Below-Ground

Root phenotyping is of intense interest as water and nutrients, significant components in plant growth and yield, are accessed and taken up by roots (Gregory et al. 2009; Zhu et al. 2011). The difficulties of visualising and measuring roots within soil are many. As John Weaver observed nearly 90 years ago, ‘There is no easy method of uncovering the root system, and unless one is willing to spend considerable time and energy, and exercise a great deal of patience, it is better not to begin’ (Weaver 1926).

Because of the importance of root biology, root phenotyping methods are rapidly evolving. Conventionally, analysis was by invasive or destructive methods such as excavation and then washing and weighing, measuring or scanning, but these are not compatible with the phenomics approach. More recently, non-destructive higher-throughput methods have been developed including visible light imaging through transparent growing media, X-ray computed tomography and NMR.

22.3.3.1 Visible Light

Plants can be grown with their roots in transparent gels, and multiple 2D images combined to generate 3D phenotyping (Clark et al. 2011; Iyer-Pascuzzi et al. 2010). The importance of this 3D analysis is emphasised (Zhu et al. 2011) because variability has been observed between root distributions of different barley varieties (Hargreaves et al. 2009).

22.3.3.2 X-Ray Computed Tomography

Visible light imaging of the roots relies on a clear and uniform growth medium, but this does not reflect the heterogeneous nature of soil. This has been tackled by the use of X-ray computed tomography for root phenotyping in soil or other granular substrates. Advances in the sensitivity of X-ray computed tomography (CT) to a resolution of less than 500 nm have allowed even fine roots to be visualised in soil in pots (Tracy et al. 2010), but throughput remains limited.

22.3.3.3 Nuclear Magnetic Resonance Imaging

Nuclear magnetic resonance (NMR) has potential for some specific aspects of root phenotyping and has been successfully used in combination with positron emission tomography to monitor root growth activity in soil substrates (Jahnke et al. 2009). However, it is limited by cost, throughput and the presence of paramagnetic metal ions in soils, such as manganese and iron, which reduce image resolution (Gregory et al. 2003; Perret et al. 2007).

22.3.4 Field Testing

To gather phenotype data on crops grown over large areas, sensors mounted to satellites or airplanes are used to capture hyperspectral images (visible, near infrared and thermal infrared). From these images a range of parameters can be estimated including canopy biomass accumulation, leaf nitrogen content and canopy temperature. Attempts have been made to quantify more complex phenotypes, such as disease development in wheat fields (Franke and Menz 2007; Mirik et al. 2011). However, at this stage, only severe infestations can be measured with any accuracy due to the insufficient spatial resolution of current technology. Depending on the geographic location, temporal resolution can be a major limitation when using satellite imagery, as images are only captured when the satellites are above the field, which was once every 16 days in the study of Mirik et al. (2011).

When greater spatial resolution is required, as is the case with small plant breeding plots, images must be taken closer to the crop canopy. This can be performed using handheld devices or by mounting sensors to vehicles or even small unmanned aircraft (Jones et al. 2009). Several studies have used canopy reflectance data to estimate the agronomic performance of field plots, including in barley (Fetch et al. 2004); however, in most instances, the predictions are significantly influenced by the environment, soil type, plant growth stage and the genotype, suggesting that further technological advances are required before it can be widely adopted. A relatively new technology in field-based phenotyping is the use of light curtains, which consist of a vertical panel of light beams that run either side of the plant rows to measure the plant profile. In maize, improved accuracy in biomass predictions was reported when a combination of canopy reflectance and light curtains was used (Montes et al. 2011).

The challenge remains to develop technologies that will enable root phenotyping in the field. One possibility is with quantitative DNA assays developed to identify crop pathogens in field soils (Ophel-Keller et al. 2008) which have been adapted to assess wheat and barley root development (McKay 2010), but phenotyping by this method is limited.

22.4 Statistical Analysis

Plant phenomics produces extremely large datasets, often with millions of data points per experiment. However, research published to date has tended to focus on utilising the high throughput, consistency and time-dependent capabilities of phenomics platforms more than this extensive range of data. This approach has certainly proven valuable (e.g. Hartmann et al. 2011; Harris et al. 2010), but capturing the full value of phenomics requires more advanced approaches.

Multiparametric statistical analysis techniques such as principal component analysis and multivariate regression analysis may be more suitable tools with which to mine these extensive datasets. These techniques use sophisticated computational techniques to identify informative parameters, and because of the wide range of phenotypes examined and the lack of preconception about which might be relevant, such approaches can often be used to develop accurate and reliable prediction models.

A detailed description of multiparametric analysis is outside the scope of this chapter; instead, see Eberius and Lima-Guerra (2009). However, it is essential to be aware that strong statistical support should be a key part of any plant phenomics research team if the full power of the phenomics approach is to be realised.

22.5 Applications

22.5.1 In General Plant Science

As with other aspects of plant science, many developments in whole-plant phenomics have focused on the model species *Arabidopsis thaliana*, which provides a straightforward system for whole-plant phenomics. In particular, its relatively small size simplifies the process of screening large numbers of plants, while its largely two-dimensional growth habit (at least prior to inflorescence development) is amenable to image capture and analysis.

Both commercial and custom-built platforms have been developed for high-throughput *Arabidopsis* phenomics. The former include the LemnaTec Scanalyzer HTS, which has been successfully used to develop robust models of growth and biomass in response to genotype and was also shown to be able to detect more subtle phenotypes than would have been measurable without automation and image processing (Arvidsson et al. 2011).

Among the custom-built phenomics platforms, two of the most established are GROWSCREEN at the Jülich Plant Phenotyping Centre and PHENOPSIS at INRA in Montpellier. GROWSCREEN has been used on both *Arabidopsis* and tobacco to study responses to changes in illumination and nutrient availability, as well as screening for stress-tolerant genotypes (Walter et al. 2007). Projects carried out using PHENOPSIS have included the identification of an *Arabidopsis* accession which displayed minimal reduction in growth rate in response to a range of soil water deficits (Granier et al. 2006).

22.5.2 In Crop Plants

While many of the technical developments in plant phenomics have been achieved in the *Arabidopsis* field, plant phenomics does differ from most other -omics technologies in that much research has bypassed such model species. Instead, it has been carried out directly on commercially important plant species, in particular cereal crops. Part of this is no doubt in recognition that discoveries in model plants (particularly *Arabidopsis*, being a member of the Brassicaceae) often do not correspond to a similar biology in cereal crops of the family Poaceae; therefore, it makes sense, where possible, to experiment directly on these commercially important species instead.

Previously, this benefit in relevance has been outweighed by the obstacles to experimentation in cereals, such as their larger size, more complex biology and generally more challenging genetics. Recent advances in high-throughput sequencing and genotyping, combined with ever more powerful bioinformatics hardware and algorithms, are now overcoming these barriers, making it much more feasible to work directly with these species of interest.

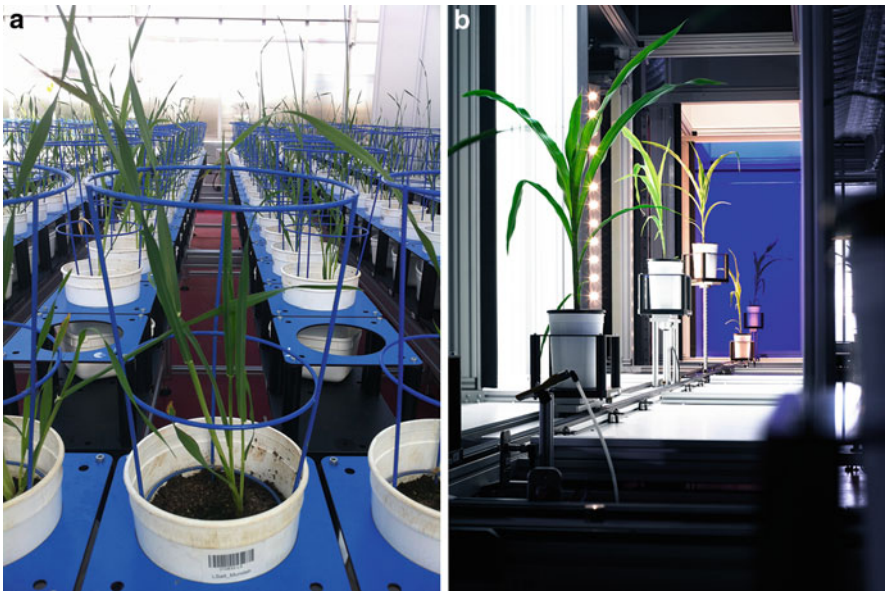

Fig. 22.2 The imaging chambers at The Plant Accelerator, from *left* to *right*: thermal infrared, visible light, short-wave infrared (soil), short-wave infrared (shoot) and fluorescence (image courtesy of LemnaTec GmbH)

22.5.2.1 Research

Probably, the most widespread current platform for crop plant phenomics research is the LemnaTec Scanalyzer 3D system (http://www.lemnatec.com). This has been installed at plant research facilities around the world including The Plant Accelerator in Australia and combines automated plant transport on conveyor belt systems to a range of imaging chambers (Fig. 22.2). Using the Scanalyzer, plants are typically phenotyped once every 1 or 2 days, although more or less frequent imaging is possible.

One common crop phenomics research application is that of trait dissection: the study in isolation of sub-traits which contribute to much more complex (and, typically, poorly heritable) traits such as abiotic stress tolerance. The high numbers of plants which can be screened, the frequent phenotyping and the reduction in biological noise due to repeated measurements on the same plants all mean that whole-plant phenomics is ideally suited to this approach. Several commercially important traits such as yield, drought resistance and salinity tolerance are all being successfully tackled with trait dissection studies (Rajendran et al. 2009; Sirault et al. 2009; Berger et al. 2010).

22.5.2.2 Commercial Applications

High-throughput whole-plant phenotyping has been adopted by a number of major multinational breeding companies; BASF and Monsanto in particular have invested heavily in this technology, the former buying the CropDesign company with its TraitMill platform (Reuzeau et al. 2010) and the latter developing its own proprietary system. The principal commercial focus of phenomics is currently on reverse genetic screening approaches to identify genes associated with stress response, particularly low water and low nitrogen, and mainly in maize, soy, rice and cotton. There seems little doubt that companies using this technology will expand the application of their phenomics platforms into other functions, including wheat and barley breeding programmes.

22.5.3 In Barley

Many of the developmental factors and environmental stresses that influence barley productivity are potentially amenable to phenomics studies, including growth rate, flowering time, plant architecture, drought, frost, salinity and pathogen infection. To date, extensive phenomics studies using barley have only been carried out on aspects of salinity and drought tolerance.

Hartmann et al. (2011) compared the growth rates of two varieties, Barke and Morex, under well-watered and drought conditions using visible light imaging on a LemnaTec Scanalyzer 3D. In addition to the expected decrease in plant size under low water conditions, they characterised developmental differences between these two varieties, including a greater interplant variability among Morex plants than for Barke. Parent (2010) used a similar platform to characterise the drought response of ten barley varieties and was able to classify these varieties into five separate groups based on their growth characteristics in both well-watered and low water environments. Characteristics measured during this project included relative and absolute growth rates, final leaf area and changes in timing of the switch from vegetative to reproductive development.

Harris et al. (2010) used the Scanalyzer 3D to characterise responses to salinity. They demonstrated quantitative and qualitative differences between the effects of salt stress on the growth rate and transpiration efficiency (biomass per unit transpiration) of Mundah and Keel, showing clear genetic contributions to these traits. Sirault et al. (2009) used the alternative technique of infrared thermography to measure elevations in leaf temperature among barley seedlings grown under saline conditions. These researchers confirmed a high correlation between temperature elevation and stomatal conductance, suggesting that thermography may be applicable as a rapid screen for salinity-tolerant varieties of barley.

Conclusion

The technological and methodological breakthroughs arising from genomics research over the last decade have provided a rich source of knowledge for plant physiologists and have inspired the development of automated high-throughput phenotyping systems. These are now enabling new applications in plant phenomics, benefitting everything from fundamental research through to applied breeding programmes.

Plant phenomics technology not only permits the phenotyping of thousands of plants in a project, it also allows researchers to study an immensely broad range of phenotypes, many of which would have been unmeasurable with conventional approaches. Furthermore, many of the available platforms are well suited to direct research in crop species like barley, reducing the need for experimentation in, and extrapolation of conclusions from, model plant species. When coupled with the ever-growing range of genomics resources in barley and other crop plants, phenomics has the potential to enable a full systems biology approach directly in these commercially valuable species. It is already being used by a number of major breeding and agricultural biotechnology companies in both their discovery and breeding pipelines.

The widespread adoption of high-throughput, data-intensive plant phenomics in contrast to low-throughput, hypothesis-driven plant phenotyping will not be without its challenges. Not least among these will be the need for much greater statistical involvement in project design, implementation and analysis. Without this, many of the benefits of the complex and diverse data generated by phenomics technologies will not be realised. Just as has been experienced in other -omics fields, there will be an increasing focus on computer-based research. At the same time, field data will continue to play an integral role, as the increasingly detailed phenotypic models generated will require validation in real production environments.

It is true that plant phenomics is still a young field and the technology and analytical approaches are relatively immature. Yet these will develop over the next few years and, as this happens, we will increasingly see the power of the phenomics approach to uncover whole new aspects of plant physiology and genetics.

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References

Arvidsson S, Pérez-Rodríguez P, Mueller-Roeber B (2011) A growth phenotyping pipeline for Arabidopsis thaliana integrating image analysis and rosette area modeling for robust quantification of genotype effects. New Phytol 191:895–907. doi:10.1111/j.1469-8137.2011.03756.x

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol 59:89–113. doi:10.1146/annurev.arplant.59.032607.092759

Berger B, Parent B, Tester M (2010) High-throughput shoot imaging to study drought responses. J Exp Bot 61(13):3519–3528. doi:10.1093/jxb/erq201

Blackburn GA (2007) Hyperspectral remote sensing of plant pigments. J Exp Bot 58(4):855–867. doi:10.1093/jxb/erl123

Blum A (2004) The physiological foundation of crop breeding for stress environments. In: Proceedings of the world rice research conference, Tsukuba, Japan, November 2004. International Rice Research Institute, Manila, pp 456–458

Cavanagh C, Morell M, Mackay I, Powell W (2008) From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. Curr Opin Plant Biol 11:215–221. doi:10.1016/j.pbi.2008.01.002

Ceccarelli S, Grando S (1996) Drought as a challenge for the plant breeder. Plant Growth Regul 20:149–155

Chan WL, Jason D, Daniel MM (2007) Imaging with terahertz radiation. Rep Prog Phys 70 (8):1325

Clark RT, MacCurdy RB, Jung JF, Shaff JE, McCouch SR, Aneshansley DJ, Kochian LV (2011) Three-dimensional root phenotyping with a novel imaging and software platform. Plant Physiol 156:455–465. doi:10.1104/pp. 110.169102

Cuin TA, Parsons D, Shabala S (2010) Wheat cultivars can be screened for NaCl salinity tolerance by measuring leaf chlorophyll content and shoot sap potassium. Funct Plant Biol 37:656–664

Eberius M, Lima-Guerra J (2009) High-throughput plant phenotyping – data acquisition, transformation and analysis. In: Edwards D (ed) Bioinformatics tools and applications. Springer, Berlin, pp 259–278

Fetch TG, Steffenson BJ, Pederson VD (2004) Predicting agronomic performance of barley using canopy reflectance data. Can J Plant Sci 84(1):1–9

Franke J, Menz G (2007) Multi-temporal wheat disease detection by multi-spectral remote sensing. Precis Agric 8(3):161–172. doi:10.1007/s11119-007-9036-y

Furbank RT, Tester M (2011) Phenomics – technologies to relieve the phenotyping bottleneck. Trends Plant Sci 16(12):635–644. doi:10.1016/j.tplants.2011.09.005

Golzarian MR, Frick RA, Rajendran K, Berger B, Roy S, Tester M, Lun DS (2011) Accurate inference of shoot biomass from high-throughput images of cereal plants. Plant Methods 7:2. doi:10.1186/1746-4811-7-2

Granier C, Aguirrezabal L, Chenu K, Cookson SJ, Dauzat M, Hamard P, Thioux J-J, Rolland G, Bouchier-Combaud S, Lebaudy A, Muller B, Simonneau T, François Tardieu F (2006) PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in Arabidopsis thaliana permitted the identification of an accession with low sensitivity to soil water deficit. New Phytol 169:623–635. doi:10.1111/j.1469-8137.2005.01609.x

Gregory PJ, Hutchison DJ, Read DB, Jenneson PM, Gilboy WB, Morton EJ (2003) Non-invasive imaging of roots with high resolution X-ray micro-tomography. Plant Soil 255:351–359. doi:10.1023/A:1026179919689

Gregory PJ, Bengough AG, Grinev D, Schmidt S, Thomas WTB, Wojciechowski T, Young IM (2009) Root phenomics of crops: opportunities and challenges. Funct Plant Biol 36:922–929

Hargreaves C, Gregory P, Bengough A (2009) Measuring root traits in barley (*Hordeum vulgare* and ssp. *spontaneum*) seedlings using gel chambers, soil sacs and X-ray microtomography. Plant Soil 316:285–297. doi:10.1007/s11104-008-9780-4

Harris BN, Sadras VO, Tester M (2010) A water-centred framework to assess the effects of salinity on the growth and yield of wheat and barley. Plant Soil 336(1–2):377–389. doi:10.1007/s11104-010-0489-9

Hartmann A, Czauderna T, Hoffmann R, Stein N, Schreiber F (2011) HTPheno: an image analysis pipeline for high-throughput plant phenotyping. BMC Bioinf 12:148. doi:10.1186/1471-2105-12-148

Iyer-Pascuzzi AS, Symonova O, Mileyko Y, Hao Y, Belcher H, Harer J, Weitz JS, Benfey PN (2010) Imaging and analysis platform for automatic phenotyping and trait ranking of plant root systems. Plant Physiol 152:1148–1157. doi:10.1104/pp. 109.150748

Jahnke S, Menzel MI, van Dusschoten D, Roeb GW, Bühler J, Minwuyelet S, Blümler P, Temperton VM, Hombach T, Streun M, Beer S, Khodaverdi M, Ziemons K, Coenen HH, Schurr U (2009) Combined MRI-PET dissects dynamic changes in plant structures and functions. Plant J 59:634–644. doi:10.1111/j.1365-313X.2009.03888.x

Jansen M, Gilmer F, Biskup B, Nagel KA, Rascher U, Fischbach A, Briem S, Dreissen G, Tittmann S, Braun S, De Jaeger I, Metzlaff M, Schurr U, Scharr H, Walter A (2009) Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in Arabidopsis thaliana and other rosette plants. Funct Plant Biol 36(11):902–914. doi:10.1071/FP09095

Jones HG, Serraj R, Loveys BR, Xiong L, Wheaton A, Price AH (2009) Thermal infrared imaging of crop canopies for the remote diagnosis and quantification of plant responses to water stress in the field. Funct Plant Biol 36:978–989. doi:10.1071/FP09123

Klose R, Penlington J, Ruckelshausen A (2009) Usability study of 3D Time-of-Flight cameras for automatic plant phenotyping. In: Proceedings of 1st international workshop on computer image analysis in agriculture, Potsdam, Germany

Maes WH, Steppe K (2012) Estimating evapotranspiration and drought stress with ground-based thermal remote sensing in agriculture: a review. J Exp Bot 63(13):4671–4712. doi:10.1093/jxb/ers165

Malone M (2011) High-throughput phenotyping – taking crop biotechnology to the next level. PhenoDays 2011, Wageningen, The Netherlands. http://www.phenodays.com/videos/michael-malone.html

McKay A (2010) Root analysis. Ground Cover (GRDC newsletter) 86 Collaboration Supplement. http://www.grdc.com.au/director/events/groundcover?item_id=7F86D9C8DA3A695B2-EB114792D5FC14F&article_id=80AD3F30F81CADCEF097012B29AFA129

Mirik M, Jones DC, Price JA, Workneh F, Ansley RJ, Rush CM (2011) Satellite remote sensing of wheat infected by wheat streak mosaic virus. Plant Dis 95(1):4–12. doi:10.1094/PDIS-04-10-0256

Montes JM, Technow F, Dhillon BS, Mauch F, Melchinger AE (2011) High-throughput non-destructive biomass determination during early plant development in maize under field conditions. Field Crops Res 121:268–273. doi:10.1016/j.fcr.2010.12.017

Munns R, James RA, Sirault XRR, Furbank RT, Jones HG (2010) New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. J Exp Bot 61(13):3499–3507. doi:10.1093/jxb/erq199

Omasa K, Hosoi F, Konishi A (2007) 3D lidar imaging for detecting and understanding plant responses and canopy structure. J Exp Bot 58(4):881–898. doi:10.1093/jxb/erl142

Ophel-Keller K, McKay A, Hartley D, Herdina D, Curran J (2008) Development of a routine DNA-based testing service for soilborne diseases in Australia. Aust Plant Pathol 37:243–253. doi:10.1071/AP08029

Parent B (2010) Drought tolerance of barley parental lines. The Plant Accelerator pilot project report. http://plantaccelerator.org.au/publications/PilotProjectReport0030.pdf

Perret JS, Al-Belushi ME, Deadman M (2007) Non-destructive visualization and quantification of roots using computed tomography. Soil Biol Biochem 39:391–399. doi:10.1016/j.soilbio.2006.07.018

Rajendran K, Tester M, Roy SJ (2009) Quantifying the three main components of salinity tolerance in cereals. Plant Cell Environ 32:237–249. doi:10.1111/j.1365-3040.2008.01916.x

Reuzeau C, Pen J, Frankard V, de Wolf J, Peerbolte R, Broekaert W, van Camp W (2010) TraitMill: a discovery engine for identifying yield-enhancement genes in cereals. Plant Gene Trait 1(1). doi:10.5376/pgt.2010.01.0001

Roy SJ, Tucker EJ, Tester M (2011) Genetic analysis of abiotic stress tolerance in crops. Curr Opin Plant Biol 14:1–8. doi:10.1016/j.pbi.2011.03.002

Scholes JD, Rolfe SA (2009) Chlorophyll fluorescence imaging as tool for understanding the impact of fungal diseases on plant performance: a phenomics perspective. Funct Plant Biol 36:880–892

Segal E, Kushnir T, Mualem Y, Shani U (2008) Water uptake and hydraulics of the root hair rhizosphere. Vadose Zone J 7(3):1027–1034

Sirault XRR, James RA, Furbank RT (2009) A new screening method for osmotic component of salinity tolerance in cereals using infrared thermography. Funct Plant Biol 36:970–977

Tracy SR, Roberts JA, Black CR, McNeill A, Davidson R, Mooney SJ (2010) The X-factor: visualizing undisturbed root architecture in soils using X-ray computed tomography. J Exp Bot 61:311–313. doi:10.1093/jxb/erp386

Tracy SR, Black CR, Roberts JA, Mooney SJ (2011) Soil compaction: a review of past and present techniques for investigating effects on root growth. J Sci Food Agric 91:1528–1537

Van Tunen A (2011) Paradigm shifts in plant breeding: automated phenotyping combined with modern genetics. PhenoDays 2011, Wageningen, The Netherlands. http://www.phenodays.com/videos/arjen-van-tunen.html

Wai Lam C, Jason D, Daniel MM (2007) Imaging with terahertz radiation. Rep Prog Phys 70:1325

Walter A, Scharr H, Gilmer F, Zierer R, Nagel KA, Ernst M, Wiese A, Virnich O, Christ MM, Uhlig B, Jünger S, Schurr U (2007) Dynamics of seedling growth acclimation towards altered light conditions can be quantified via GROWSCREEN: a setup and procedure designed for rapid optical phenotyping of different plant species. New Phytol 174(2):447–455. doi:10.1111/j.1469-8137.2007.02002.x

Weaver JE (1926) Root development of field crops. McGraw-Hill, New York

Zhu J, Ingram PA, Benfey PN, Elich T (2011) From lab to field, new approaches to phenotyping root system architecture. Curr Opin Plant Biol 14:310–317. doi:10.1016/j.pbi.2011.03.020

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