Chapter 5
Barley

Jutta Schulze

Abstract Barley (*Hordeum vulgare* L.) is a widely adapted cereal crop with an extremely wide geographic distribution throughout the world. It finds great use for animals as a feed and for humans as a grain, especially as the source for malt for the brewing industry. In recent times, there is considerable interest in the nutritional properties of barley due to the discovery of the cholesterol-lowering effect of β-glucan, a cell wall polysaccharide. Exploitation of genetic diversity in the primary and secondary gene pool of barley using DNA-based technologies has yielded interspecific crosses with improved grain properties, malting quality and resistance to biotic and abiotic stresses. The significant achievements regarding introgression of alien genes include the genes *Rym14(Hb)*, *Rym16(Hb)* and *Ryd4(Hb)* which were introgressed from *Hordeum bulbosum* conferring resistance to BaMMV, BaYMV and BYDV in barley. Significant advances in genetic engineering of barley have been obtained, and strategies for establishment of regenerative cell and tissue culture systems as well as for development of DNA delivery techniques have been formulated. Lately, a huge potential has been realised in barley grains to produce pharmaceutical proteins like oral vaccines, growth supplements and food additives which are being exploited in a commercial scale. Nevertheless, several problems still remain like the strong genotype dependency of barley transformation protocols, transformation efficiency, transgene stability and public acceptance. The review focuses on all these issues and elaborates achievements made in the last two decades in genetic enhancement of barley using different alien gene transfer approaches.

Keywords Abiotic stress • Barley • Biopharming • Disease resistance • Field trials • Gene transfer • Wide hybridisation

J. Schulze (✉)
Department of Plant Biology, Technical University of Braunschweig, Humboldtstr. 1, D-38106 Braunschweig, Germany
e-mail: Jutta.Schulze@tu-bs.de

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5.1 Introduction

Crop plants derived from their ancestors in a long process from preferential harvest of wild plants to directed selection of plants with good performance resulted in systematic breeding for desirable agronomic traits. The process, which started approximately 10,000 years ago along with settlement of men, resulted in high yielding cultivars to meet the demands of the ever-increasing population of the world. However, elite cultivars are vulnerable to a wide range of pressures, thus plant breeders must constantly respond to adapt and improve crops.

More than half of the food consumed by mankind is based on the major cereals, viz. maize, wheat, rice and barley, since these crops are the main sources of plant carbohydrates and proteins (FAO 2012). Thus, cereals are substantial for production of animal feed, starch, flour, sugar, oils, processed foods, malt, alcoholic beverages, gluten and renewable energy (Edgerton 2009). The "Green Revolution" and intensification of crop management led to an increase in productivity of these crops until the 1980s (Hedden 2003). However, in the last two decades, growth rates of yields slowed down due to declining resources of arable land and water, deteriorating soil conditions as a result of environmental degradation and climate change (Schmidhuber and Tubiello 2007; Mba et al. 2012) as well as due to limitations in the germplasm pool (McIntosh 1998; Prada 2009). It is estimated that feeding nine billion people in 2030 would demand raising overall food production by some 50 % between 2005/2007 and 2030 (Beddington 2010; Wegner and Zwart 2011). Thus, there is an urgent need for new approaches and technologies and also for generating new varieties to meet that dramatic increase (Edgerton 2009; Phillips 2010).

Barley (*Hordeum vulgare* L.), the number four in the world's cereal crops with respect to production quantity, yield (t/ha) and acreage (FAOSTAT), is a widely adapted plant with an extremely wide geographic distribution. In 2011, the estimated world production of barley was 134 million tons (FAOSTAT). The largest use is for animal as well as human food, especially as the source for malt for the brewing industry. However, in recent time there is considerable interest in the nutritional properties of barley due to the discovery of the cholesterol-lowering effect of β-glucan, a cell wall polysaccharide found in barley (Newton et al. 2011).

H. vulgare is well studied regarding genetics, cytogenetics and genomics. Cultivated barley is self-pollinated and diploid with $2n = 2x = 14$ chromosomes, and its genome size is about 5.5×10^9 bp with 80 % highly repetitive DNA. Besides the availability of numerous germplasm collections, a lot of data have been accumulated in recent time concerning molecular markers, genomic DNA sequences, full-length cDNAs and expressed sequence tags (ESTs) supplemented by voluminous studies on genomics, proteomics and metabolomics which are accessible in different databases (Sreenivasulu et al. 2008). Strikingly, sequencing the genome of barley has become a realistic task (Schulte et al. 2009). In parallel, tremendous efforts in cereal transformation technology were made allowing now comprehensive functional analysis of genes. Combining these developments, barley is now a model plant for the *Triticeae* (Saisho and Takeda 2011), which is reflected by numerous

reports on transgenic barley in very recent time. This chapter attempts to summarise achievements made in alien gene transfer in barley with emphasis on agronomic traits as well as fundamental research.

5.2 Introgression of Alien Genes by Wide Hybridisation

A key to successful barley protection and high yield is the constant genetic improvement in this crop. Barley breeding has a long and prosperous history with respect to enhancement in resistance levels and yield, and extensive progress has been achieved in the last few decades (Friedt et al. 2011). However, the modern elite cultivars show a relatively low level of genetic diversity, and the loss of important traits like resistances have led to genetic uniformity due to gene erosion. Nevertheless, barley is challenged by a range of biotic and abiotic stresses, and continuously, (1) new sources emerged due to climate change and (2) the pathogens respond with a rapid adaptation (Gregory et al. 2009; Pautasso et al. 2012). The traditional way for introgression of new genes or for the combination of desired traits is sexual recombination combined with phenotypic selection and analysis of the progeny. However, classical breeding procedures are seriously challenged if sources of natural resistance to pathogens are rare as in case of barley yellow dwarf virus (BYDV) (Ordon et al. 2005; Kosová et al. 2008).

5.2.1 Wide Crosses

Wild or related species of cultivated crops represent large resources for desirable genes. In attempts to exploit a broader germplasm resource for improvement of barley, wild relatives and landraces were re-evaluated (Pickering and Johnston 2005; Steffenson et al. 2007; Newton et al. 2010; Nevo and Chen 2010). Various aspects of introgressing genes from wild barley into domesticated barley have been comprehensively covered previously (Fedak 1989). The ancestor of domesticated barley, *H. vulgare* ssp. *spontaneum*, which belongs to the primary gene pool, shows no incompatibility in crossings, while hybridisation with *Hordeum bulbosum*, the only member of the secondary gene pool, is difficult (von Bothmer et al. 2003). The use of these wild relatives as a source in breeding programmes can be realised by using the embryo rescue technique. However, strong crossability barriers exist to the 30 species of *Hordeum* belonging to the tertiary gene pool (Pickering and Johnston 2005). Successful reports on transfer of resistances against several threats like powdery mildew (Pickering et al. 1995), leaf rust (Pickering et al. 1998), Septoria speckled leaf blotch (Toubia-Rahme et al. 2003) and barley yellow mosaic virus (Ruge et al. 2003; Ruge-Wehling et al. 2006) from *H. bulbosum* into *H. vulgare* are available.

Recently, the development of a set of introgression lines (Ils) for barley was reported with each IL carrying a single introgression of the exotic *H. vulgare* ssp.

spontaneum accession ISR42-8 in the genetic background of the elite spring barley cultivar Scarlett. The set was generated by backcrossing, selfing and marker-assisted selection. In order to illustrate the applicability of the spring barley ILs, the lines were used for verification of quantitative trait loci (QTLs) for field resistance against powdery mildew (*Blumeria graminis* f. sp. *hordei* L.) and leaf rust (*Puccinia hordei* L.) (Schmalenbach et al. 2008). In addition, QTLs were verified in these ILs affecting malting quality parameters (Schmalenbach and Pillen 2009). It was validated that wild barley alleles at the chromosome 1H QTL reduced overall malting quality, whereas wild barley alleles at the chromosome 4H QTL improved the malting quality parameters compared to the control genotype Scarlett (March et al. 2012). Furthermore, a set of 110 putative ILs containing chromatin introgressed from *H. bulbosum* L. into cultivated barley has been identified using a high-copy number retrotransposon-like PCR marker. Introgressed chromatin from *H. bulbosum* was confirmed and genetically located in 88 of these lines using 46 of the EST-derived PCR markers (Johnston et al. 2009). Notably, resistance to stem rust, caused by *Puccinia graminis* f. sp. *tritici*, which is an important disease in *H. vulgare*, was transferred from *H. bulbosum* into cultivated barley (Fetch et al. 2009). Moreover, the successful transfer of *Ryd4Hb*, a novel resistance gene introgressed from *H. bulbosum* into barley and conferring complete and dominant resistance to the barley yellow dwarf virus, has to be highlighted (Scholz et al. 2009).

5.2.2 Somatic Hybridisation

An alternative approach to transfer genes from distant species or from wild relatives is hybridisation of somatic cells to circumvent sexual incompatibilities. Despite a large number of somatic hybrids that have been produced in a number of crop plants (Liu et al. 2005), there are only a very few reports on protoplast fusion in barley. Application of this technique for barley improvement is severely limited due to difficulties encountered with plant regeneration from barley protoplasts as will be discussed later. The formation of hybrid calli between *H. vulgare* L. and *H. bulbosum* was described, but plant regeneration was not reported (Funatsuki et al. 1994).

Protoplast fusion experiments in barley and rice yielded one intergeneric somatic hybrid plant, which resembled with rice in its morphology. Cytological studies revealed large chromosomes from barley and small chromosomes from rice. Southern hybridisation with a fragment of the *tryptophan B* (*trpB*) gene detected barley-specific and rice-specific bands. Furthermore, novel mitochondrial and chloroplast sequence rearrangements were also reported that were not detected in either of the parents (Kisaka et al. 1998). Fusion between barley and carrot protoplast was also carried out in an attempt to transfer cold and salt tolerance from barley into carrot. Morphology of the three regenerated plants closely resembled that of the parental carrot plants (Kisaka et al. 1997).

5.3 Application of Biotechnological Approaches

Biotechnological methods can improve the efficiency of barley breeding since (1) they offer the opportunity to access to additional gene pools, (2) allow the insertion of individual genes and (3) reduce time-consuming conventional techniques of crossing and backcrossing. Transfer of genes, which cannot be introduced via sexual hybridisation due to pre- and post-zygotic incompatibilities, requires gametic or somatic cells competent for regeneration. Thus, one prerequisite for the production of fertile transgenic plants is a totipotent target tissue and, second, methods to deliver DNA into these cells. Both the processes, which have been developed independently, have to be combined to provide highly efficient, cost-effective and easy to handle protocols as a powerful tool for crop improvement programmes and for analysis of gene function.

5.3.1 Establishment of Regenerative Systems in Barley

With respect to cell biology, single cells like protoplasts are an ideal target for direct DNA uptake. As described for the other cereals, protoplasts in barley can be isolated in large quantities from the leaves, roots and stems. Nevertheless, only in rare and irreproducible cases protoplasts divided to form callus. Thus, rapidly growing embryogenic cell suspensions were used as alternative source, but plant regeneration as a prerequisite for generating transgenic plants could not established as a routine method (Jähne et al. 1991; Funatsuki et al. 1992; Davey et al. 2005).

In parallel, multicellular explants were analysed to establish highly regenerative systems. Leaves derived from in vitro as well as ex vitro grown plants, which are the preferential source for callus initiation with subsequent plant regeneration in dicots, gave only a poor or no response in barley as well as for all other members of the cereals. Thus, other explants like mature seeds, isolated mature embryos, tissues derived from young seedlings, immature embryos, inflorescences, nodes and roots were evaluated, and a large number of reports are available (reviewed in Schulze 2007). In the second half of the 1980s, a general consent emerged that immature embryos are the most suitable explants. Additionally, anthers and microspores, extensively analysed for the production of haploid barley plants, reveal embryogenic capacity thus being an excellent target for gene transfer since homozygous transgenic plants can be developed more rapidly (Devaux and Kasha 2009).

Nevertheless, irrespective of the explant source used, the regeneration potential in barley is strongly affected by several factors like genotype, medium composition, phytohormones and growing conditions of the donor plants. There are a number of reports, which demonstrate that tissue culture ability and green plant regeneration is under genetic control (Bregitzer and Campbell 2001; Tyankova and Zagorska 2001). Further, an ever-increasing number of reports deal with improvements of regenerability by optimising or adapting media components for elite

genotypes. Consequently, some progress has been made, for example, by substitution of 2,4-D by picloram (Przetakiewicz et al. 2003; Chauhan and Kothari 2004) or dicamba (Halámková et al. 2004; Aguado-Santacruz et al. 2011), supplement of thidiazuron (Schulze 2007; Gubisová et al. 2012), increasing cupric sulphate (Bregitzer et al. 1998a; Nuutila et al. 2000) or incorporating ethylene precursor 1-aminocyclopropane 1-carboxylic acid or adding ethylene antagonist silver nitrate (Jha et al. 2007; Tyagi and Dahleen 2011). However, these manipulations also could not affect the genotype dependency with respect to regeneration. A tissue culture system for barley that appeared to be largely genotype independent seems to be the ovule culture technique (Holm et al. 1995), which however requires highly specialised resources and skills. A recent transcript-derived marker barley map based on ESTs was used to locate QTL for barley green plant regeneration and identify candidate genes, which include a ferredoxin-nitrate reductase and genes involved in hormone response and synthesis in cell division and the cell cycle (Tyagi et al. 2010). The identification of these genes should be the next step to manipulate regeneration ability in barley.

5.3.2 Development of DNA Delivery Techniques

The use of *Agrobacterium*-mediated gene transfer which got quickly established for numerous dicots in the 1980s was not that successful with cereals, since wounding of differentiated cereal tissues does not lead to the wound response-induced dedifferentiation in wound-adjacent cells (Potrykus 1990). Thus, numerous other methods for DNA transfer into the regenerative competent cells were developed for cereals which were also applied to barley. Amongst these methods, direct DNA transfer into protoplasts was easily achieved due to the absence of the cell wall; however, the first report on successful culture and selection of transgenic barley callus lines was published only in 1991 (Lazzeri et al. 1991), and it took another 4 years that fertile transgenic barley plants were generated via the protoplast approach using polyethylene glycol-mediated DNA uptake (Funatsuki et al. 1995; Kihara et al. 1998), followed by electroporation (Salmenkallio-Marttila et al. 1995) and microinjection in zygote protoplasts (Holm et al. 2000). Alternative methods were employed to circumvent difficulties of barley cell culture like imbibing of embryos in DNA (Töpfer et al. 1989), electrophoresis of DNA into germinating seeds (Ahokas 1989) and macroinjection of DNA into floral tillers or application of plasmid-DNA to stigmas (Mendel et al. 1990). However, no evidences for stable transformation were presented. Altogether, at the end of the 1980s, it emerged that those cells, which are transformable, are unable to regenerate and tissues like immature embryos with a high regenerative capacity lack methods to transform. A breakthrough was, however, the development of the particle gun (Klein et al. 1987), which enables direct transfer of DNA into regenerable tissues. The feasibility of microprojectile bombardment to transfer and express foreign DNA in barley cells was demonstrated by Mendel et al. (1989) and Kartha et al. (1989). Consequently,

the generation of fertile, transgenic barley was achieved using different targets like immature embryos (Wan and Lemaux 1994; Ritala et al. 1994; Hagio et al. 1995; Koprek et al. 1996; Jensen et al. 1996), microspores (Jähne et al. 1994; Leckband and Lörz 1998; Shim et al. 2009), embryogenic callus from immature embryos (Cho et al. 1998; Manoharan and Dahleen 2002) and mature embryos (Um et al. 2007) as well as in vitro shoot meristematic cultures derived from germinated seedlings (Zhang et al. 1999).

Successful transformation of cereals with *Agrobacterium* could be achieved due to the known advantages and also utilisation of hyper-virulent *Agrobacterium* strains as well as vectors containing extra copies of *vir* genes which together have helped in overcoming the restricted compatibility of the *Poaceae*. The progress made with rice (Hiei et al. 1994) and maize (Ishida et al. 1996) paved the way for barley also. Tingay et al. (1997) first demonstrated the suitability of *Agrobacterium tumefaciens*-mediated transformation for barley using immature embryos, and the method was optimised with regard to in vitro culture conditions (Trifonova et al. 2001; Bartlett et al. 2008) and factors influencing wounding and coculture (Shrawat et al. 2007). Additionally, the feasibility of other targets like embryogenic callus (Wang et al. 2001), ovules (Holme et al. 2006) and androgenetic pollen (Kumlehn et al. 2006) for *Agrobacterium*-mediated transformation was also explored resulting in a constantly growing number of reports in terms of stable expression of alien genes. A comparative analysis of transgenic barley plants generated via particle bombardment as well as via *Agrobacterium*-mediated DNA delivery clearly revealed a higher transformation efficiency, low-copy integration (between one and three copies in 100 % of the lines) and a stable inheritance of the T-DNA as a simple Mendelian trait for the *Agrobacterium*-derived lines (Travella et al. 2005). Experimental results of a large-scale study using Southern analysis indicated vector backbone integration in 48 % of the transgenic lines derived from *Agrobacterium*-mediated transformation of immature embryos in barley as described for other plants (Lange et al. 2006). Likewise, the twin T-DNA strategy based on transformation with an *A. tumefaciens* vector containing two adjacent T-DNAs thus enabling segregation of the selectable marker gene away from the gene of interest was also successfully applied for barley. The method represents a powerful approach for elimination of the selectable marker gene (Matthews et al. 2001).

In most cases barley transformation yielded in the regeneration and selection of heterozygous transgenic plants. Nevertheless, the doubled haploid (DH) lines are important tools for breeding and analysis of gene function. Basing on a huge knowledge in barley androgenesis, protocols were provided for inducing homozygosity in transgenic barley lines using microspore culture (Ritala et al. 2005). Importantly, it was demonstrated, that this is also a practicable and efficient approach for production of selectable marker-free, homozygous transgenic barley plants (Coronado et al. 2005).

Recently, a substantial increase in the transformation rate of barley was achieved due to a detailed study including comparison of *Agrobacterium* strains under diverse experimental conditions and the use of relatively high concentrations of L-cysteine and acetosyringone as supplements for cocultivation. This powerful protocol

enables a transformation efficiency up to 86.7 stable transgenics per 100 immature embryos inoculated with *A. tumefaciens* which was never described before (Hensel et al. 2008). Besides that, the integrated T-DNA copy numbers are typically low, the inheritance of the transgenes is according to the Mendelian rules, and the protocol is applicable for other genotypes and breeding lines also.

5.3.3 Targeted Expression of Alien Genes

The significant progress achieved in the last decade in barley transformation is correlated to elucidation of mechanisms that control transgene expression with respect to (1) strength, (2) cell and tissue specificity, (3) developmental specificity and (4) environmental effects. Thus, the choice of the promoter is of primary importance. Whereas, for development and optimisation of gene transfer methods in barley, the widely used constitutive promoters from the cauliflower mosaic virus gene (*35S*), rice actin 1 gene (*Act1*) and maize polyubiquitin gene (*ubi-1*) mainly were employed (Wan and Lemaux 1994; Jähne et al. 1994; Tingay et al. 1997), numerous specific promoters were isolated and introduced in cereals transiently and stably (Hensel et al. 2011). The first report for barley was a homologous approach to functionally validate the expression of barley high-pI α-amylase gene promoter and signal peptide-coding region fused to a hybrid bacterial thermostable (1,3-1,4)-β-glucanase. Nearly 75 % of grains harvested from primary transformants synthesised the gene of interest (Jensen et al. 1996). Likewise, the expression of a cloned fragment from the seed-specific β-amylase gene from barley was confirmed using the β-glucuronidase gene (*gus*) in a homologous system, and GUS activity was found in the subaleurone endosperm during seed maturation (Okada et al. 2000). Endosperm-specific expression during grain maturation in transgenic barley was detected analysing the barley B1 hordein (*Hor2-4*) and D-hordein (*Hor3-1*) promoters (Cho et al. 1999b, 2002; Furtado et al. 2009), a rice glutelin B1 (*GluB-1*) promoter (Patel et al. 2000) and two high-molecular-weight glutenin (*HMW-Glu*) promoters (Schünmann et al. 2002; Zhang et al. 2003; Furtado et al. 2009). Besides that, in a study with the green fluorescent protein gene (*gfp*) as a reporter, the wheat early methionine (Em) promoter was evaluated which maintained endosperm-specific expression in barley (Furtado and Henry 2005) suggesting its ability as a strong promoter to direct transgenes in specific tissues of barley. Furthermore, the oat globulin *AsGlo1* promoter region (960 bp) and a 251 bp fragment were used to produce transgenic barley. The mechanism of its specificity is different from that observed in glutelin and prolamin promoters due to a novel interrupted palindromic element (Vickers et al. 2006). The promoters of two rice genes (*OsPR602* and *OsPR9a*) fused to *gus* were also analysed in stably transformed barley, which displayed activity in early grain development with the strongest expression in endosperm transfer cells during the early stages of grain filling (Li et al. 2008). Likewise, the promoter of *ZmMRP-1*, a maize endosperm transfer cell-specific transcriptional activator, which plays a central role in the regulatory pathways controlling cell differentiation,

was introduced in barley. GUS activity was detected in the developing modified aleurone layer which indicates that the promoter responds to functional, transport-related signals (Barrero et al. 2009).

Targeted gene expression is a critical step to combat fungal pathogens in barley and any other crop. The promoter of the *Lem2* gene of barley, which encodes a lectin-like protein that is strongly upregulated by salicylic acid and is preferentially expressed in lemma, palea and coleoptile, was analysed. Promoter/*gfp* reporter constructs revealed cell- and development-specific expression of *gfp* in lemma/palea, glumes, coleoptile, auricle and ligule (Abebe et al. 2006). Another option in this regard is the promoter of a lipid transfer protein (*ltp6*) which has been cloned from barley. Different promoter deletion constructs were examined using *gfp,* and strong expressions in the ovaries and pericarp epidermis and during embryogenesis and germination were detected reflecting the expression pattern of the native gene therefore being suited for targeted disease resistance (Federico et al. 2005).

Recently, the promoter of the germin-like protein (*GER4*) was identified, which is involved in the pathogen-associated molecular pattern of barley leaf epidermis attacked by the powdery mildew fungus *Blumeria graminis* f. sp *hordei. GER4c* promoter responds with a high transcript dosage due to pathogen attack and seems to be a powerful tool to engineer disease resistance in cereals (Himmelbach et al. 2010).

Besides the availability of numerous specific promoters useful for controlling gene expression in cereals and establishment of high-throughput *Agrobacterium*-mediated transformation protocols, the demand for binary vectors has increased since these enable an easy insertion of promoters, effector sequences and selectable markers. For this purpose a set of modular binary vectors has been developed (Himmelbach et al. 2007).

5.4 Achievements in Transgene Technology in Barley

In the last decade tremendous progress has been made in genetic transformation of barley. There are numerous reports of both applied and basic nature, which imply that barley transformation is now optimised and a routine. Consequently, barley has emerged as a model plant of the *Triticeae* tribe (Saisho and Takeda 2011) and proves that elucidation of gene function is no longer restricted to the dicots.

5.4.1 Disease Resistance

Like other crops, barley is adversely affected by bacteria, fungi and viruses which cause a great variety of diseases. Depending on several factors like climatic conditions and crop protection measures adopted, losses due to pests are still high globally. In a study published in 2006, the global total potential loss for wheat varied from 26 to 29 %, whereas for maize and rice, it was 31–37 %, no figures were given

for barley (Oerke 2006). Nevertheless, a few data are available for individual countries and pests. Taking the example of barley yellow dwarf virus infection on yield and malting quality of barley in the USA, various aspects have been comprehensively examined and losses up to 40 % have been reported (Edwards et al. 2001). Economic losses for barley resulting from impacts of Fusarium head blight (FHB) were assumed to be up to 55 % for North Dakota and Minnesota from 1998 to 2000 (Nganje et al. 2001). Recently, in an assessment on the losses caused by diseases alone to the Australian barley industry, it was estimated that pathogens caused an average loss of 19.6 % of the average annual value of the barley crop in the decade 1998–1999 to 2007–2008 (Murray and Brennan 2010).

5.4.1.1 Fungus Resistance

A large part of research in barley genetic engineering is aimed at increasing fungal resistance. The first transgenic approach to increase fungal resistance in barley was transformation of the stilbene synthase gene of *Vitis vinifera*, resulting in the expression of the phytoalexin resveratrol capable of detoxifying fungal toxins. Pathological experiments indicated an enhanced resistance of T_1 plants in a detached leaf assay after inoculation with *Botrytis cinerea* (Leckband and Lörz 1998). In an effort to combat stem rust caused by *Puccinia graminis* f. sp. *tritici* in barley, the *Rpg1* gene for resistance to stem rust was introduced in a highly susceptible cultivar. A single copy of the gene conferred resistance against stem rust, and progenies from several transformants segregated in a 3:1 ratio for resistance/susceptibility, as expected. Therefore, it was demonstrated that the functional *Rpg1* gene isolated by map-based cloning coded for stem rust resistance (Horvath et al. 2003). On contrary, recently it was reported that transgenic barley lines overproducing functional RPG1 protein due to insertion of four or five copies responded with susceptibility to stem rust probably caused by the failure to degrade the RPG1 protein (Chai et al. 2012). Similarly, the maize *Rp1-D* gene, which confers race-specific resistance against *Puccinia sorghi* isolates containing a corresponding *avrRp1-D* avirulence gene, was inserted into barley but did not result in novel resistances when these plants were challenged with isolates of barley leaf rust *P. hordei* (Ayliffe et al. 2004).

The interaction of barley with the powdery mildew fungus *Blumeria graminis* f sp. *hordei* is presently the subject of intense research. Here, the polymorphic *Mla* locus harbouring race-specific resistance (R) genes is involved. To gain insights into *Mla*-mediated resistance, epitope-tagged Mla-variants, whose expression is driven by native regulatory sequences, were used for generation of transgenic barley lines. The findings show a reversible and salt concentration-dependent distribution of the intracellular MLA proteins in soluble and membrane-associated pools. The data demonstrate that *Rar1* encoding a intracellular Zn^{2+} binding protein positively controls steady levels of MLA resistance proteins and leads to accumulation of MLA6, thus enabling effective resistance (Bieri et al. 2004). Furthermore, stable over-expression of the constitutively activated barley RAC/ROP protein, RACB, reveals the enhancement of susceptibility to powdery mildew concluding that RACB might be involved in signalling in response to biotic stress (Schultheiss et al. 2005).

Besides that, the involvement of *HvRBOHF2*, a respiratory burst oxidase homologous NADPH oxidase gene, as well as *HvBI-1*, a BAX inhibitor with function in negative control of programmed cell death, was analysed for the interaction of barley and the powdery mildew fungus. Transgenic barley plants were developed with stably knocked down expression of *HvRBOHF2* which were unable to contain wound-induced cell death and revealed developmental alterations from the three-leaf stage onward. The results suggest that RBOHF2 is required for normal development of penetration resistance to the fungus (Proels et al. 2010). Moreover, barley plants carrying an *HvBI-1* RNA interference (RNAi) construct having lower levels of *HvBI-1* respond with less susceptibility to powdery mildew than wild-type plants accompanied by enhanced resistance to penetration by *B. graminis* f. sp. *hordei* at the cellular level (Eichmann et al. 2010). In contrast, transgenic barley plants over-expressing the cell death-regulating BAX inhibitor *HvBI-1* display suppression of defence response and resistance to *B. graminis* f sp. *hordei*; however, young seedlings were more resistant to *F. graminearum*. The authors concluded that the life cycle of the fungus influences the outcome of the effect of *HvBI-1* (Babaeizad et al. 2009).

Barley plants over-expressing the *HvBI-1* were a valuable tool to investigate the relationship between the fungus *Piriformospora indica* and barley. The endophytic fungus induces root resistance against head blight caused by *Fusarium culmorum* and also systemic resistance to powdery mildew via an unknown mechanism. Cytological and molecular evidences suggest that *P. indica* needs dead host cells for proliferation which progresses as the tissues mature. The expression level of the cell death regulator *HvBI-1* influences development of *P. indica* in barley. Fungal proliferation was remarkably reduced in the transgenic lines indicating that *P. indica* requires host cell death for proliferation (Deshmukh et al. 2006).

Another approach being explored is the use of pathogenesis-related (PR) proteins known to be associated with degradation of structural components of pathogenic filamentous fungi. Transgenic barley plants were generated by co-bombardment with two plasmids, one containing a rice (*Oryza sativa* L.) chitinase gene (*chi11*) and another carrying a rice thaumatin-like protein gene (*tlp*). From T_1 plants expressing both the proteins, T_3 homozygous lines were developed that co-express both antifungal proteins (Tobias et al. 2007). These lines when tested for many years exhibited reduced *Fusarium* head blight (FHB) incidence (Dahleen et al. 2011). The fungus also produces the mycotoxin deoxynivalenol (DON) which inhibits protein synthesis and is harmful to humans and animals and therefore reduces crop quality. A strategy to reduce DON accumulation in the grains focussed on introduction of *Tri101*, which encodes a 3-OH trichothecene acetyltransferase that converts DON to a less toxic acetylated form in barley. T_3 and T_4 progenies of three independent transgenic lines with *Tri101* showed a reduction in DON concentration (Manoharan et al. 2006). These lines were backcrosses and two of the resultant lines consistently showed a 40 % reduction in DON (Dahleen et al. 2011).

Another serious disease in barley is root rot caused by *Rhizoctonia solani* and *R. oryzae*. Transgenic barley liners have been developed to ubiquitously express a codon-optimised 42-kDa endochitinase *cThEn(GC)* from *Trichoderma harzianum* (Wu et al. 2006). Chitinases from this soil fungus effectively break down chitin, the main constituent of fungal cell walls of mature hyphae, conidia, chlamydospores

and sclerotia. The transgenic lines of barley displaying resistance to *Rhizoctonia* were analysed in field to monitor possible side effects of the genetic modification compared to the parental cultivar Golden Promise. Moreover, to assess influence of normal genotypic variation a second cultivar Baronesse was included in the study. Using parallel transcriptome and targeted metabolome profiling, as well as nontargeted metabolite fingerprinting, the data exhibited that cultivar-specific differences remarkably exceeded the effects caused by the transgene expression (Kogel et al. 2010).

A further interesting attempt to engineer disease resistance in barley against fungal plant pathogens is the use of antifungal peptides from insects. The suitability of metchnikowin, an antimicrobial peptide from *Drosophila melanogaster*, was evaluated for its resistance properties against damaging fungi. The transformed barley harbouring the metchnikowin gene showed increased resistance to powdery mildew, FHB and root rot. Additionally, accumulation of metchnikowin was also detected in plant apoplastic space specifying that the insect signal peptide is functional in monocotyledons (Rahnamaeian et al. 2009).

5.4.1.2 Virus Resistance

One of the most serious viral diseases of cereals worldwide is barley yellow dwarf (BYDV). Since sources of natural resistance to this virus are rare (Ordon et al. 2005; Kosová et al. 2008), the use of virus-derived transgenes was amongst the early approaches. Constructs containing the coat protein of several isolates of BYDV together with selectable markers were used resulting in some resistant barley plants (Wan and Lemaux 1994; McGrath et al. 1997); however, unfortunately resistance was not stable. Further experiments succeeded in transformation of barley with transgenes encoding an hpRNA derived from BYDV-PAV polymerase sequences, and one-third of the independently transformed lines exhibited very high resistance to BYDV-PAV (Wang et al. 2000). This was followed by the transfer of transgenes derived from BYDV and cereal yellow dwarf virus (CYDV) in an elite Australian barley cultivar. While there was considerable variability amongst the virus levels in different transgenic lines developed, some of the plants containing transgenes showed reduced virus symptoms (Wang et al. 2001).

The feasibility of using the barley "eukaryotic translation initiation factor 4E" (*Hv-eIF4E*), which was identified as a candidate for resistance gene function by physical mapping, was also analysed. It could be shown that *Hv-eIF4E* confers multiallelic recessive *Bymovirus* resistance in barley (Stein et al. 2005).

5.4.2 Abiotic Stresses

Significant yield losses are caused in barley by various abiotic stresses including drought, flooding, salinity, wind and temperature extremities and climate change in envisaged to increase the problem further (Gregory et al. 2009). Physiological,

biochemical and molecular approaches have been used to dissect the response of plants to abiotic stresses describing effectors, regulatory genes and gene networks emphasising the pivotal role of transcription factors (Nakashima et al. 2009).

Approximately 40 % of agriculturally used area is covered by acid soils strongly limiting productivity. Due to acidity, aluminium is solubilised which rapidly inhibits root growth and thereafter water as well as nutrient uptake. A malate transporter from wheat (*ALMT1*) enabling malate efflux was introduced in barley and the transgenic lines when evaluation for response to aluminium (Al) stress demonstrated a high level of aluminium tolerance in hydroponic culture as well as on acid soils (Delhaize et al. 2004). Further analysis on relation between aluminium resistance and phosphorus nutrition in wild-type and transgenic plants expressing *TaALMT1* revealed a higher efficiency of the transgenics in taking up phosphorus on acid soil. In addition, a higher root growth, shoot biomass and grain yield were observed for the *TaALMT1*-plants in comparison to the control when grown up to maturity on the same soil (Delhaize et al. 2009). In another study, Li et al. (2010) found increased aluminium resistance in roots of transgenic barley over-expressing *Phalaris coerulescens* thioredoxin gene (*PTrx*) (Li et al. 2010).

Um et al. 2007 generated transgenic barley plants containing cDNA from *Arabidopsis* nucleoside diphosphate kinase 2 (*AtNDPK2*). They observed 10 % reduction in membrane damage in the transgenic plants caused by methyl viologen which indicated the expression of *AtNDPK2*. Similarly, an alfalfa aldo-keto reductase (*MsALR*) which can detoxify lipid peroxide degradation products was over-expressed in barley to eliminate toxic reactive aldehyde products from cells after oxidative stress. The cellular stress response of the transgenic plants was investigated in transient assay estimating damaged cells microscopically using fluorochromes and determining chlorophyll as well as carotenoid content (Nagy et al. 2011). In all cases transgenic plants outperformed controls after applying stress.

Recently, two dehydration-responsive proteins (DREBs) from wheat were analysed for their potential to modify transcriptional regulation of drought and cold stress in barley (Morran et al. 2011). Constitutive over-expression of *TaDREB2* and *TaDREB3* resulted in stable transformed plants which responded significantly better to drought and cold stresses compared to the controls. However, these showed negative impacts on developmental parameters like stunted growth, dwarfism, delayed flowering and smaller spikes. In contrast, it was observed that the drought-stress-inducible *ZmRab17* promoter is quickly and strongly activated by drought causing little or no adverse developmental traits (Morran et al. 2011). Besides that, the feasibility of the transcription factor *Osmyb4* from rice characterised as a central point of a large transcriptional network was also evaluated for modulation of stress response in barley. Progeny of transgenic lines harbouring *Osmyb4* under control of the *Arabidopsis* cold-inducible promoter *cor15a* was exposed to freezing, and the damage was determined through analysis of chlorophyll fluorescence parameters. During germination pronounced differences were observed concerning higher vigour to hypoxia combined with cold stress compared with the controls. These data support an involvement of *Osmyb4* in flooding tolerance and in alleviation of germination under adverse environmental conditions (Soltész et al. 2012).

5.4.3 Improvement of Product Quality and Plant Productivity

5.4.3.1 Polysaccharides

A large part of applied research is focussed on alteration of processing quality of barley grains since the starchy endosperm contributes to about 80 % of the total grain weight. The linear polysaccharides (1,3-1,4)-β-glucans are the major constituent of endosperm cell walls in barley. Thus, enzymatic mobilisation of endosperm storage constituents requires degradation of these cell walls for a high and efficient use of barley grains for feed and malting.

In early attempts barley was manipulated to express a codon-optimised bacterial thermostable (1,3-1,4)-β-glucanase to improve the digestibility of the grains in monogastric animals like poultry. About 75 % of grains from primary transformants synthesised thermostable (1,3-1,4)-β-glucanase and inheritance of transgene expression was reported in scutellum and aleurone of germinating seeds (Jensen et al. 1996, 1998; Horvath et al. 2000; Xue et al. 2003). Likewise, a fungal xylanase gene under the control of an endosperm-specific promoter from cereal storage protein was introduced in barley to produce plant cell wall polysaccharide-hydrolysing feed enzymes in the endosperm to replace the later addition of microbial produced xylanases to the feed thus reducing the costs (Patel et al. 2000).

An important aspect regarding malting quality in barley is increasing thermostability of enzymes, since in malting and brewing industries, the grains are exposed to temperatures above 70 °C. In this direction, a mutant thermostable β-amylase gene generated by site-directed mutagenesis was used to design transgenic barley plants. An increase in thermostability by 11.6 °C compared to the original enzyme was obtained which was stably transmitted to progeny (Kihara et al. 2000). Similarly, a gene encoding for a thermotolerant fungal endo-(1,4)-β-glucanase (Nuutila et al. 2002) and the heat-stable alkalophilic *Bacillus* α-amylase (Tull et al. 2003) was also introduced in barley resulting in an enhancement of α-amylase activity by 30–100 % compared with the control.

In another approach the wheat thioredoxin *h* gene (*wtrxh*) driven by a seed-specific promoter was over-expressed in barley to gain insight in its putative role in germination and seedling development. The results demonstrated an increased activity of a starch-branching enzyme of the endosperm, pullulanase specifically hydrolysing α-1,6-linkages in starch (amylopectin) during germination and seed development (Cho et al. 1999a). In addition, these plants displayed enhanced root and shoot growth in the presence of 2 mM sodium selenite suggesting that overexpression of thioredoxin *h* could be a tool for application in the remediation of polluted soils (Kim et al. 2003).

Further aspects of interaction between starch metabolism and plant development were studied by antisense downregulation of the barley limit dextrinase inhibitor (LDI). Transgenic barley plants were developed to investigate the function of LDI. In homozygous antisense lines, an increased LD activity was observed in developing and germinating seeds accompanied by unpredicted pleiotropic effects on

numerous enzyme activities, reduced numbers of the small B-type starch granules and reduced amylose relative to amylopectin levels (Stahl et al. 2004). Another transgenic approach using RNAi-mediated silencing was addressed to the starch-branching enzymes, SBE IIa and SBE IIb, to define structure of amylose and amylopectin in the barley endosperm. The data suggested that a reduction in the expression of both SBEs was necessary to significantly increase amylose content in comparison to the wild types (Regina et al. 2010). Very recently, the simultaneous suppression of all starch-branching enzyme genes (*SBE I*, *SBE IIa* and *SBE IIb*) using a chimeric RNAi hairpin was described. Carciofi et al. (2012) succeeded in generation of barley with amylose-only starch granules which were irregularly shaped. The grains of the transgenic lines germinated like the controls displayed comparable high yield, but growth was delayed suggesting an important physiological role of amylopectin (Carciofi et al. 2012).

Of late, the (1,3-1,4)-β-D-glucan, a major constituent of the cell wall of cereals and grasses, has gained renewed interest of plant scientists due to its beneficial effects on human health and as a valuable source of fermentable sugars for bioethanol production (Newton et al. 2011). Investigations were concentrated on increasing the (1,3-1,4)-β-D-glucan levels by over-expression of barley cellulose synthase-like family (*CslF*) cDNAs under control of an oat globulin promoter or a constitutive promoter. An enhanced amount of 80 % (1,3-1,4)-β-D-glucan in grains of transgenic barley was obtained in case of the endosperm-specific promoter, whereas gene expression driven by the constitutive promoter resulted in sixfold higher levels of (1,3-1,4)-β-D-glucan in vegetative organs and similar levels in grains compared with the control (Burton et al. 2011). A further analysis was conducted to explore the role of α-glucosidase in germinating barley grains. In seedlings harbouring an RNA interference silencing cassette for *HvAgl97*, α-glucosidase was lowered up to 50 %. The findings indicate that the α-glucosidase *HvAGL97* is the major endosperm enzyme catalysing the conversion of maltose to glucose but is not required for starch degradation in contrast to results from biochemical assays with glucosidase inhibitors (Stanley et al. 2011).

5.4.3.2 Proteins

With respect to manipulation of seed storage proteins, a cDNA encoding the γ-zein protein of maize driven by an endosperm cell-specific promoter was used to determine deposition pattern and impact on grain properties. In transgenic barley, an accumulation of nearly 2 % of γ-zein of the total grain nitrogen was reported corresponding to 4 % of the total protein fraction. However, no effects on grain texture like hardness or vitreousness were described (Zhang et al. 2003). A very interesting approach is the production of a therapeutic protein in barley grains. Since barley is a major constituent of feed for domestic animals, its suitability as a source for oral vaccination against porcine diarrhoea caused by F4-positive enterotoxigenic *Escherichia coli* (ETEC) strains was explored. A protective immune response against the disease is inducible by F4 fimbriae or FaeG. Transgenic barley was

designed expressing the F4 fimbrial adhesin FaeG in a glycosylated form in the endosperm up to 1 % of total soluble protein. The recombinant protein was resistant to storage and simulated digestive conditions. In addition, glycosylation did not negatively influence immunogenicity since erFaeG was able to induce F4 fimbria-specific antibodies in mice (Joensuu et al. 2006).

The alteration of the amino acid composition of barley is important to improve the feeding quality of its grains and also to avoid the use of large-scale protein supplements derived from soybean or microbes. Efforts were undertaken to increase the content of essential amino acids, especially lysine, threonine and methionine in barley. Lange et al. (2007) aimed at selective suppression of C-hordein synthesis, the storage protein with the lowest nutritional value by an antisense approach. From the 35 primary transformants, five lines were selected for comprehensive analysis using SDS-PAGE and reverse phase HPLC. Their data demonstrated a relative reduction in the content of C-hordeins combined with a relative rise in the synthesis of other storage proteins in the mature grain. An increase was found in lysine, threonine and methionine content (16, 13 and 11 %) indicating antisense-mediated suppression of C-hordein synthesis as a promising approach (Lange et al. 2007). The data were confirmed by a transcriptomic analysis of one of the antisense C-hordein lines using a grain-specific cDNA microarray (Hansen et al. 2007). More recently, another strategy to manipulate lysine content in barley grains has also been successfully applied. The key enzyme involved in the regulatory step for lysine biosynthesis dihydrodipicolinate synthase (*dapA*) from *E. coli* was employed. Analysis revealed T_1 lines with enhanced level of lysine in leaves as well as T_2 lines with higher amount in seeds relatively to the wild type (Ohnoutkova et al. 2012).

5.4.3.3 Micronutrients

Research activities have been increasingly focussed in barley on combating micronutrient deficiency with regard to the plant as well as to the subsequent consumer using this plant product. In an attempt to increase phosphate uptake in barley plants, a high-affinity phosphate transporter was over-expressed in barley, but this did not enhance phosphate uptake in transgenic plants (Rae et al. 2004). In contrast, over-expression of an *Arabidopsis* zinc transporter (*AtZIP1*) resulted in a rise of short-term zinc uptake after zinc deficiency and seed zinc content thereby improving its nutritional quality (Ramesh et al. 2004). Very recently, the genetic modification of barley for improvement of phytase activity was reported applying the cisgenesis concept. Phytases are essential enzymes for the sequential release of phosphate groups from phytic acid thereby providing bioavailable phosphate which otherwise could not be used from monogastric animals. Enhanced phytase activity up to 2.6- to 2.8-fold was found in the seeds from lines homozygous for the insert. Besides that, the activity levels were stable over the three generations assayed (Holme et al. 2012).

5.4.3.4 Plant Productivity

For acceleration of plant development, the *vhb* gene encoding *Vitreoscilla* haemoglobin (VHb) known to improve cellular respiration and efficient energy generation during oxygen-limited growth was inserted in barley. Nevertheless, constitutive *vhb*-expressing plants failed to fulfil the expectations (Wilhelmson et al. 2007). In an attempt to develop early flowering barley plants, the natural early flowering time allele Cape Verde (Cvi) of Cryptochrome2 (*AtCRY2-Cvi*) gene from an *Arabidopsis* was employed. Seeds from T_1 plants were evaluated which recorded more than 25 days earlier flowering and day-length insensitivity as compared to the controls (El-Din et al. 2011).

Zalewski et al. (2010) concentrated on silencing the expression of cytokinin oxidase/dehydrogenase (*HvCKX1*) applying RNAi-based technology to elucidate the function of the gene in barley. The authors succeeded in generating more than 50 lines from which nearly 80 % displayed significantly reduced CKX activity in bulked samples of their T_1 roots. A positive relationship between enzyme activity and plant productivity was determined, reflected as the yield, the number of seeds per plant and 1,000 grain weight. Consequently, decreased CKX activity led to a higher plant yield and root weight (Zalewski et al. 2010). Furthermore, silencing of *HvCKX2* resulted in different phenotypes depending on the transformation method. *Agrobacterium*-mediated gene transfer yielded silenced lines with higher productivity, whereas biolistic silenced lines exhibited low productivity and disturbances in plant development (Zalewski et al. 2012).

5.4.4 Barley Grains as a Bioreactor

Cereal grains offer an excellent opportunity for production of recombinant pharmaceutical proteins since they were bred to accumulate and store large amounts of carbohydrates and proteins. Molecular breeding in cereal crops with respect to expression level, protein authenticity, downstream processing and purification as well as regulatory issues has been comprehensively covered previously (Ramessar et al. 2008; Hensel et al. 2011). Barley is of great importance because of it being a self-pollinator, unable to generate fertile hybrids with related species and wide adaptation with an extremely wide geographic distribution.

The applicability of barley to produce heterologous proteins was initially demonstrated using a protein-engineered thermostable (1,3-1,4)-β-glucanase and a fungal xylanase (Nuutila et al. 1999; Horvath et al. 2000; Patel et al. 2000; Xue et al. 2003). Consequently, this potential was explored for substances, for which traditional production methods are expensive, inefficient and laborious. Thus, an antibody-fusion protein used to detect HIV-1 in human blood by causing rapid agglutination was expressed in barley. Schünmann et al. (2002) succeeded in high-level expression of an antiglycophorin single-chain antibody fused to an epitope of the HIV virus in seeds of barley, which can substitute the SimpliRED™ diagnostic reagent. Additionally, the yield in barley (150 µg/g of regent per gramm) exceeded

amounts expressed in transgenic tobacco leaves and potato tubers which were evaluated in parallel (Schünmann et al. 2002). Besides that, transgenic barley plants comprising genes for production of human antithrombin III, α1-antitrypsin, lysozyme, serum albumin and lactoferrin were reported (Stahl et al. 2002). Transgenic barley plants expressing human lactoferrin (*hLF*) were also described from other groups. Western blot analysis of leaf tissue from T_0 plants documented the expression of the recombinant human lactoferrin (Kamenarova et al. 2007), whereas Tanasienko et al. (2011) provided proof for the presence of the gene *hLF* fragment in leaves of T_0 plants by PCR.

Eskelin et al. (2009) and Erlendsson et al. (2010) presented further convincing evidences that transgenic barley seeds can be utilised as a bioreactor. The successful expression of both the recombinant full-length and the 45-kDa fragment of human collagen-type I α-1 chain (*rCla1*) in barley seeds was obtained screening three promoters. The proteins were further targeted to the endoplasmic reticulum to enhance the expression levels of recombinant proteins as previously shown (Horvath et al. 2000). The glutelin promoter was superior in yielding 45 mg recombinant protein per kg dry seeds in the best lines compared to 15 mg/kg caused by the ubiquitin promoter (Eskelin et al. 2009). Moreover, the Orfeus™ expression system developed by ORF Genetics (Reykjavik, Iceland) was used to produce recombinant human Flt3 ligand in barley grains. For that purpose, the cDNA of human Flt3 ligand, a growth factor necessary for proliferation and differentiation of stem cells, with an HQ_6-tag under control of the hordein promoter was used. High expression of biologically active Flt3 ligand with a yield comparable to prokaryotic production was reported (Erlendsson et al. 2010).

Consequently, this huge potential relying on plant-based recombinant protein production, in general and in cereal-based production in particular, is exploited commercially by several companies like Maltagen Forschung GmbH (Andernach, Germany), ORF Genetics (Reykjavik, Iceland) and Ventria Bioscience (Fort Collins, USA) and is reflected in patents (Stahl et al. 2009). Thus, the barley endosperm is an efficient bioreactor for pharmaceutical proteins like cytokines, oral vaccines, growth factors and food additives.

5.4.5 Elucidation of Gene Function

The enormous progress made in barley transformation in the last decade together with the dramatic advances in genome research due to new technologies as well as an ever-increasing number of data accessible in public databases (Sreenivasulu et al. 2008) has enabled the functional characterisation of candidate genes identified in functional genomic studies. Comprehensive determination of gene function via over-expression or reduction of gene expression up to the knockout of plant genes is no longer restricted to the model dicot *Arabidopsis thaliana*. Thus, insights in metabolic and regulatory networks possibly linked to agronomically important traits become important. Table 5.1 summarises examples for the elucidation of gene function made in barley so far.

Table 5.1 Elucidation of gene function in barley

Promoter specificity/ coding sequence	Effect	Reference
Wheat HMWGLU-1 D1 antisense *SnRK1* protein kinase	Promoter activity in seeds and anthers 50 % pollen: arrested at binucleate stage, contains little or no starch, and is non-functional, male sterility	Zhang et al. (2001)
HvGAMYB (transcription factor in barley aleurone and anthers)	Active in early anther development, over-expression led to decrease in anther size, male sterility	Murray et al. (2003)
jekyll (expressed in barley grain nucellar projection tissue)	RNAi: decelerates autolysis and cell differentiation within nurse tissues, no function of nuclear projection as main transport route for assimilates, irregular and small-sized seeds	Radchuk et al. (2006)
LOX2:Hv:1 with and without the chloroplast targeting signal	Over-expression: higher levels of jasmonic acid for lines with elevated levels of LOX-100 in chloroplasts and in cytoplasma, respectively	Sharma et al. (2006)
TaMSH7 mismatch repair gene	RNAi: results in reduced seed set	Lloyd et al. (2007)
Short Vegetative Phase (SVP)-like MADS-box genes	Ectopic expression: inhibition of spike development, floral reversion, florets at the base of the spike, delay of head emergence, inhibition of floral meristem identity	Trevaskis et al. (2007)
PpENA1 (sodium-pumping ATPase from the bryophyte *Physcomitrella patens*)	Over-expression: marked increase in levels of free amino acids, organic acids, and salicylic acid and in some sugars and fatty acids	Jacobs et al. (2007)
HvABA8′OH1 (ABA catabolism)	RNAi: reduced expression results in higher levels of ABA and increased dormancy	Gubler et al. (2008)
Whirly1 (encodes a nucleic acid-binding protein)	RNAi: plastid located Whirly1 functions primarily in RNA metabolism rather than as a DNA-binding protein	Melonek et al. (2010)

5.5 Problems and Prospects

5.5.1 Genotype Dependency

Despite significant progress achieved in the last decade in transfer of alien genes into *H. vulgare* using biotechnological tools, the strong genotype dependency is still a key problem and still hampers routine application of gene transfer to improve traits in a desired cultivar. A highly efficient and reproducible regeneration system using immature embryos is only available for the spring variety, Golden Promise, identified in the middle of the 1980s (Lührs and Lörz 1987). The responsiveness

of this genotype to regeneration protocols is convincingly demonstrated in a summary on barley transformation up to 2007 where nearly two-thirds of all reports used only this genotype (Schulze 2007). Dahleen and Manoharan (2007) pointed out that this model genotype is suitable for improvement of transgene technology but is not suited for evaluation of transferred genes in local field conditions owing to less agronomic value. In fact, from a practical point of view, agronomically superior cultivars adapted to the region should only be used despite their recalcitrance to tissue culture methods in order to achieve gains in terms of production and quality. A few successful reports are available for commercially important barley varieties including the Finnish elite cultivar Kymppi (Ritala et al. 1994; Nuutila et al. 1999); several German spring cultivars (Koprek et al. 1996; Sharma et al. 2006); the North American cultivars, Harrington, Galena and Conlon (Cho et al. 1998; Zhang et al. 1999; Manoharan and Dahleen 2002; Manoharan et al. 2006; Tobias et al. 2007); the elite Australian barley cultivars, Schooner, Chebec and Sloop (Wang et al. 2001; Murray et al. 2004); and a Bulgarian winter barley (Kamenarova et al. 2007). However, in all these studies transformation efficiency was reported to be low. Similarly, the transformation frequency in other spring and winter genotypes that were generated was also low despite considerable improvements of protocols for *Agrobacterium*-based transformation (Hensel et al. 2008). Recently, stable transformation of commercially important varieties from Saudi Arabia (El-Din et al. 2011), from the Ukraine (Tanasienko et al. 2011) and from India (Yadav et al. 2013) was reported reflecting the need for improvements of cultivars, which can be used effectively in areas with special conditions as described for instance for the Ukraine having also marshy woodlands, forest-steppe, and steppe (Tanasienko et al. 2011).

Holme et al. (2008) reported a genotype-independent method of DNA delivery using young barley embryos derived from in vitro cultured ovules as targets for *Agrobacterium*-mediated transformation. Nonetheless, this method is not suited for high-throughput and cost-oriented transformation technology due to its sophisticated and labourious protocol. Other possible targets are in vitro shoot meristematic cultures derived from germinated seedlings which can be induced with low genotype dependency (Ganeshan et al. 2003; Sharma et al. 2004). The feasibility of these meristematic cultures characterised by proliferation of tightly packed clusters of continuously multiplying axillary and adventitious buds (Fig. 5.1) was validated for barley also (Zhang et al. 1999).

5.5.2 Transgene Insertion and Stability

Another problem in barley transformation is transgene stability. Direct DNA delivery frequently results in multicopy integration, and rearrangement of the transgene and gene silencing often has been linked to this phenomenon (Vaucheret et al. 1998; Cho et al. 1999b, 2002; Bregitzer and Tonks 2003). Convincing evidences were provided by Travella et al. (2005) comparing transgenic barley lines generated by biolistics and *Agrobacterium*-mediated method. Sixty per cent of the particle

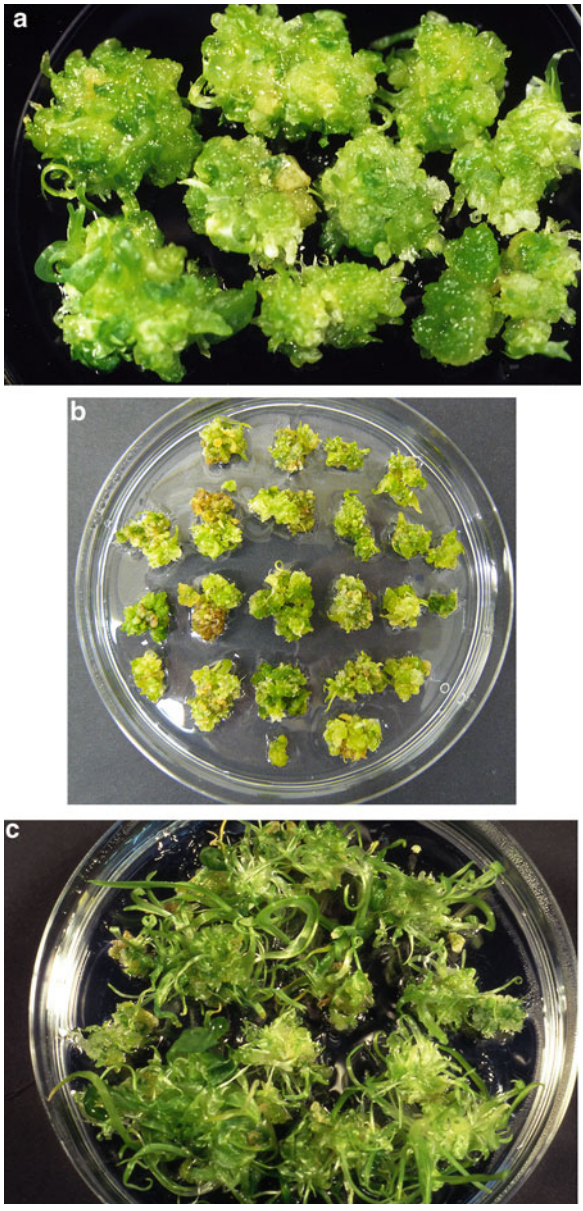

Fig. 5.1 Morphogenic response of cultures established from meristematic shoot segments from barley (*H. vulgare* cv. Lomerit) as described by Sharma et al. (2004). (**a**) Tightly packed clusters of continuously multiplying axillary and adventitious buds. (**b**) Green clumps of shoot buds on maintenance and proliferation medium 4 weeks after subculture. (**c**) Multiple shoot formation from bud clumps 2 weeks after transfer on regeneration medium

bombardment-derived lines integrated more than eight copies of the transgenes. Besides that, in all those lines extensive DNA rearrangements with multiple integrations were observed. In contrast, integration of only 1–3 copies of the transgenes with minimal rearrangements was detected in the lines produced by *Agrobacterium*-mediated method. Further, in case of *Agrobacterium*-based lines, analysis of

progeny revealed that the integrated T-DNA was inherited as a simple Mendelian trait and no silencing of the *bar* gene was observed in T_1 plants (Travella et al. 2005). The advantages of *Agrobacterium*-mediated gene transfer with respect to lower copy number for barley were also substantiated by Bartlett et al. (2008) and Hensel et al. (2008). The other possible reasons for silencing of transgenes could be promoter interference (Tobias et al. 2007) and methylation of the first untranslated exon and 5′ end of the intron in the *Ubi1* promoter complex (Meng et al. 2003).

A comprehensive study on transgene integration using fluorescence in situ hybridisation (FISH) from 19 independent barley lines revealed that transgene integration sites were found only on five of the seven barley chromosomes (Salvo-Garrido et al. 2004). Further, specific regions of the chromosomes 4H and 5H were detected containing clusters of transgene insertions, thus indicating a non-random pattern of integration. The data suggested that transgene insertions were preferentially located in gene-rich areas of the genome. A promising new tool to target a transgene to a specific locus in crops was recently presented by Shukla et al. (2009) using designed zinc-finger nucleases (ZFNs) that induce a double-stranded break at their target locus. The concomitant expression of ZFNs and delivery of a simple heterologous donor molecule resulted in precise targeted addition of a herbicide-tolerance gene at the intended locus in *Zea mays*, and genetic changes were transmitted to the progeny (Shukla et al. 2009).

Analysis of long-term stability of *gus* and *sgfp*(S65T) driven by the B1- and D-hordein promoter up to the T_9 generation revealed transgene stability in 93 % of the transgenic lines examined, while expression of *bar* under control of the maize ubiquitin promoter was found in only 60 % lines (Choi et al. 2003). Advanced generation of these lines containing the transgenes were crossed to obtain plants expressing multiple transgenes. Thus, a homozygous T_8 plant containing *gus* driven by the barley endosperm-specific B1-hordein promoter was crossed with another homozygous T_4 plant, carrying *sgfp(S65T)* driven by the barley endosperm-specific D-hordein promoter. PCR was used to monitor F_1 progeny for the transgenes *gus* and *sgfp*(S65T). Furthermore, functional expression of both transgenes was evaluated up to the F_4 generation. Localisation of transgenes by FISH revealed the same location of transgenes as in the parental plants (Choi et al. 2009).

5.5.3 Marker Gene Elimination

A requirement for the commercial use of genetically modified barley is the elimination of the selectable marker gene. For this purpose, several approaches have been evaluated. Matthews et al. (2001) investigated the twin T-DNA principle as already mentioned. An *Agrobacterium* vector comprising two contiguous T-DNAs, one with the gene of interest and the other one with the selectable marker gene, can be used to generate T_1 lines which have inserted the gene of interest and is free of the selectable gene (Matthews et al. 2001; Xue et al. 2003). This methodology is routinely used now. Another strategy aims at androgenetic generation (haploid technology)

of a segregating population of homozygous plants raised from pollen of primary transgenic barley plants produced via *Agrobacterium* infection of immature embryos. The results demonstrated that selectable marker-free homozygous transgenic plants can be efficiently generated (Coronado et al. 2005; Kapusi et al. 2013). However, even though haploid technology represents an elegant solution and accelerates time and resource efficiency of generating true-breeding, selectable marker-free transgenic barley, the major limitation is its strong genotype dependency. In both studies mentioned above, the variety Golden Promise was used which shows a poor response in pollen culture. Hence, culture conditions for immature pollen were elaborated. No promising reports on barley varieties are available which show a reasonable regenerative response of immature embryos and pollen. Therefore, the regeneration process has to be standardised for each and every genotype independently, which is a time-consuming process.

5.5.4 Field Trials and Risk Assessment

Assessment of genetically modified plants under natural field conditions is essentially required (1) to analyse transgene stability under natural environment, (2) to verify possible negative side effects of the introduced gene, (3) to monitor influence of natural genotypic variation, (4) to study ecological impacts, and (5) to evaluate the impact of environment on the expression of transgene. To analyse agronomic performance of genetically modified barley, the initial field trials were conducted in 1994 using T_2 generation of transformed plants. Compared to seed-derived Golden Promise plants, the transgenics were shorter and showed lower yield, smaller seed and a high variability amongst the individual plants (Bregitzer et al. 1998b). In another small-scale field experiment monitoring several agronomic traits, no differences were reported in the transgenic barley lines containing the *bar* gene and non-transformed control plants (Harwood et al. 1999). Yet in another study, reduced 1,000-grain weight and variable yield reductions were reported in transgene lines as compared to the Golden Promise cultivar (Horvath et al. 2001).

Field assessment also helps to adjudge the behaviour of a transgenic plant when it is taken to the natural field conditions. For instance, for the modification of the mycotoxin deoxynivalenol (DON) produced by the fungus *F. graminearum*, the transgenic T_3 and T_4 barley lines revealed a reduction of DON concentration in greenhouse test. However, this observation was not confirmed under field conditions, and possibly variations in temperature and humidity, inoculum and disease pressure could have overwhelmed the effects of *Tri101* against DON (Manoharan et al. 2006). Kogel et al. (2010) observed that cultivar-specific differences markedly exceed effects caused by the transgene expression as discussed above.

To study the level of gene flow, field trials were conducted with genetically modified homozygous barley lines harbouring the gene for neomycin phosphotransferase II in 1996 and 1997 in Finland. In these studies, while male sterile barley lines were used as recipients for pollen from the transgenics, normal male fertile

barley was also included to monitor transgene flow in normal barley. The results clearly indicated that the chance of cross-pollination to normal fertile barley varied from 0 to 7 % at 1 m distance, depending on weather conditions. However, the rate of cross-pollination declined rapidly with an increase in isolation distance; thus, in a range of 50–100 m distance, only a few seeds developed on male sterile barley due to cross-pollination from transgenic lines (Ritala et al. 2002; Nuutila et al. 2002). Studies on gene flow were also conducted under field conditions between transgenic and non-transgenic barley cv. Golden Promise in south-eastern Australia. The results indicated that outcrossing occurred at a rate of 0.005 % over a distance of less than 12 m, and therefore, the risk of gene flow between transgenic and non-transgenic barley at the field scale would be very low providing that crops were separated by a few metres (Gatford et al. 2006).

The tremendous progress made in barley genetic engineering is also reflected in the number of proposals for field trials. For the USA, 83 applications for release of transgenic barley in the period from 1993 up to 2010 were submitted. In contrast, there were only nine proposals for the European Union between 1996 and 2009 (GMO compass 2013). In all these cases the traits that have been targeted are fungal resistance, modified product characteristics and herbicide tolerance.

In Australia, currently a comprehensive research programme led by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) is underway which started in 2009 with approval of the Office of the Gene Technology Regulator (OGTR) to assess genetically engineered barley in small field plots in a 3-year test. Meanwhile six additional applications were approved (DIR094, DIR099, DIR102, DIR111, DIR112, DIR117) for release of GM barley lines containing genes for alteration of starch metabolism, for enhancement of the content of resistant starch and for improvement of nitrogen use efficiency and abiotic stress tolerance as approved by OGTR (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/map; verified February 2013). In contrast, the number of field trials with genetically modified plants in general is declining in Europe (http://www.gmo-safety.eu/news/1416.plant-research-europe-genetic-engineering-field-trials.html; verified February 2013). A small field trial with genetically engineered barley producing the enzyme phytase is being carried out between 2011 and 2015 in the Czech Republic (Notification Number B/CZ/11/2) to verify stability of the transgene *phyA* in progeny (GMO Register 2013). Similarly, field trials with transgenic barley have also been conducted very recently (between 2010 and 2013) in Denmark and Sweden (GMO Register 2013). In the USA, 11 field tests were conducted between 2011 and 2012 focussing on *Fusarium* head blight resistance, *Rhizoctonia* resistance and increased nitrogen utilisation efficiency (details available on http://www.nbiap.vt.edu/).

5.5.5 Public Acceptance

One of the major hurdles in large-scale adoption of transgenic barley is its public acceptance. Since barley is mainly used for brewing and malting industry as well as

feed for animals, the inclusion of selectable markers like antibiotic or herbicide resistance is a cause of concern and invokes debates worldwide. Marker-free transgenic plants are one of the solutions to this issue (Matthews et al. 2001; Xue et al. 2003). Further, the gene flow between the transgenic barley and its wild counterpart also remains a concern despite careful investigations on this issue (Gatford et al. 2006). Additionally, apprehensions with regard to possible health risks by consumption of end products from genetically modified barley also exist thereby forcing the industry to take a wait-and-see stance. All these concerns have sometimes resulted in acts of vandalism, for example, in Europe (Kuntz 2012) and some of the field trials for barley have also been the targets of destruction (http://www.gmo-safety.eu/news/505.destruction-barley-trial-field.html; verified February 2013).

To circumvent the problems associated with the use of artificial gene combinations, the cisgenesis concept was developed (Schouten et al. 2006). The concept implies that the genetic material introduced in a plant should originate from the plant itself or from a species being crossable with that plant; thus, the gene pool for cisgenesis is the same as for classical breeding. Additionally, sequences from selectable marker genes or vector backbone sequences have to be absent. The feasibility of the cisgenesis concept was analysed for barley also aiming at improved phosphate bioavailability in the grains which are used as feed for monogastric animals such as pigs and chickens. A barley phytase gene (*HvPAPhy_a*) was used which is expressed during grain filling, and marker-free plant lines were recovered applying the marker gene elimination method. The insertion of the genomic clone for *HvPAPhy_a* resulted in lines with enhanced activity of phytase as discussed above (Holme et al. 2012), and field trials were conducted in 2012 (GMO Register 2013, Notification Number B/DK/12/01).

5.6 Conclusions and Future Prospects

Tremendous progress has been made in alien gene transfer to barley during the last two decades, converting it from an otherwise recalcitrant crop to a model for the *Triticeae*. Advances in distant hybridisation were achieved due to development of genomic tools and molecular techniques enabling marker-assisted selection and targeted backcrossing. The exploitation of genetic diversity through extensive screening programmes evaluating related wild species and landraces for valuable agronomic traits and sources of resistance to biotic and abiotic stresses provided an important tool for the generation of lines containing introgressed segments from wild species such as *Rym14(Hb)*, *Rym16(Hb)* and *Ryd4(Hb)* derived from *H. bulbosum* and conferring resistance to BaMMV, BaYMV and BYDV in barley.

Conventional breeding, of late, has been complemented by the biotechnological approaches of alien gene transfer, and these were particularly benefited from the establishment of *Agrobacterium*-mediated genetic transformation technology in combination with significant progress in genomic research. Consequently, functional characterisation of candidate genes for targeted manipulation of specific characters

is now possible and is extensively used for fundamental and applied research in barley. Nevertheless, despite development of numerous genetically modified barley lines with improvements in product quality, composition and resistance to stresses, most of these lines are still in the laboratory or in experimental field trials. If outcomes of the analyses made in Australian field trials are positive, commercial varieties will be available in around 2020 (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/map; verified February 2013). Similarly, encouraging reports are also there from the trials being conducted in the USA. Therefore, it can be concluded that the recent developments made in distant hybridisation, standardisation of tissue culture protocols, establishment of *Agrobacterium*-mediated and other genetic transformation techniques and development in barley genomics together will pave a way for continuous development of barley cultivars having genes from more distant and alien backgrounds, widening the genetic base of existing cultivars.

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