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Transgenic Barley in Fundamental Research and Biotechnology

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ABSTRACT

Barley represents both a useful experimental model for a number of small-grain cereals as well as an agronomically important crop for feed and food production. In recent years, a vast amount of different barley genetic resources has been generated and collected worldwide. Together with these resources, the development of reliable transformation technologies has stimulated a variety of approaches to functional gene analysis and genetically engineered breeding lines. The technical details of the gene transfer process are central to the establishment of powerful transformation technology. In contemporary methods, agrobacteria are employed as naturally evolved and artificially optimized vehicles to integrate recombinant DNA into the barley genome. In addition, essentially three types of regenerable targets for stable *Agrobacterium*-based DNA-transfer are particularly useful in barley, each having specific advantages. While the use of immature zygotic embryos results in the highest efficiency of transgenic plant formation, androgenetic pollen cultures permit a more rapid production of true-breeding transgenic lines, and isolated ovules allow for the generation of transgenic plants without use of a selectable marker. Interestingly, the latter two systems are exclusively available in barley to date. In this paper, a current overview on barley transformation technologies is presented, including information on vector systems, gene transfer methods and targets, transferred coding and regulatory DNA-sequences as well as methods to generate barley without unnecessary recombinant DNA integrated in its genome. Moreover, transformation-based approaches to genetically improve barley and functionally characterise DNA-sequences associated with various aspects of crop performance are comprehensively surveyed. Further on, it is demonstrated that barley grains constitute a promising production platform for molecular farming.

Keywords: *Agrobacterium*, genetic transformation, *Hordeum vulgare*, molecular farming, resistance

CONTENTS

INTRODUCTION

Barley is the world's most ancient farmed cereal, and the fourth major cereal crop in acreage and production, surpassed only by wheat, maize and rice. Barley exhibits a broad range of adaptability, including the ability to grow at high altitudes and latitudes, under a wide range of day-length variation and marginal conditions such as salinity and limited rainfall. It grows within a considerable range of environments that vary from northern Scandinavia, to the Himalayan Mountains and monsoon paddies. Thus, barley is one of the most variable and adapted cereal crops with continued prominence in today's agriculture. The estimated world production of barley in 2005 was around 138 million metric tons (UN Food and Agriculture Organization, FAO). The majority of barley crop yields are used as animal feed because of the comparatively high protein content of the grains, particularly in the winter-type cultivars (12-15%). In turn, two-rowed spring-type barley grains for which a quite little protein content (~9%) and a high percentage of carbohydrates (65%) is characteristic are typically used as malt for the production of alcoholic beverages. In some parts of the world, barley is also used to nourish humans. The highest consumption occurs in North African countries (www.

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fao.org/AG/AGP/agpc/doc/field/other/act.htm). However, the low content of lysine and threonine is limiting its nutritional value for humans as well as for monogastric animals. In turn, barley consumption has been reported to have several health benefits, especially for obese and diabetic patients. These benefits are mainly assigned to the high content of β-glucans in barley grains (Lifschitz *et al*. 2002). Diet enriched with β-glucan results in a reduced rate of sugar absorption, a decrease of postprandial glucose and an attenuated glycemic response (Kim *et al*. 2005). Furthermore, Behall *et al*. (2004) showed that a daily diet containing 3 or 6 g barley-derived β-glucan causes a significant decrease of total cholesterol, which may result in a reduced risk of cardiovascular diseases. Although β-glucans are not desirable in malting and feed barley, it is considered to be a neutraceutical for humans in this respect. More detailed information on the nutritional profile of mature barley grains was reported by Chung *et al*. (1989).

Barley, like other crops, is prone to a number of pests, such as viruses, bacteria, fungi, and insects. Its basic susceptibility to stem rust (*Puccinia graminis*) and powdery mildew (*Blumeria graminis* f. sp. *hordei*) could so far be fairly effectively counteracted through sexual recombination with germplasm carrying naturally occuring resistance alleles. Nevertheless, comprehensive agronomical pest management including the application of fungicides is necessary to combat these major fungal pathogens. However, Bymovirus and Barley yellow dwarf (BYDV/CYDV) diseases as well as abiotic stresses for which the power of conventional measures is limited represent additional serious concerns.

Barley has a long-standing history as an excellent experimental model system representing a number of small-grain cereal species, including wheat. While the barley genome is structurally quite similar to that of other cereals, it is considerably more amenable to investigations of classical and molecular genetics mainly owing to its true diploidy.

The establishment of powerful genetic transformation systems for barley is vital for the introduction of several useful traits, such as enhanced feed and food value, improved malting and brewing qualities and increased disease resistance. In this review, we provide a current survey on technologies associated with barley transformation, e.g. information on methods and targets of gene transfer, on expression systems, on the retrieval of valuable DNA-sequences, as well as on approaches to generate transgenic barley without unnecessary recombinant DNA. Comprehensive data is presented on the functional characterisation of nucleotide sequences associated with plant performance such as grain development and responses to pathogens. Further on, based on numerous examples it is pointed out that barley grains potentially constitute a valuable production platform for molecular farming.

TRANSFORMATION TECHNOLOGIES

Target cells and methods of gene transfer

The establishment of cell culture systems that permit efficient regeneration of plants constitutes a major technical prerequisite for the development of transformation technologies. Cereals have long been considered recalcitrant in this respect, since it has been hardly possible, if not impossible, to obtain plant regeneration from differentiated leaf or root cells, as is more easily achieved in many dicotyledonous species. Therefore, the identification of totipotent cells and tissues that are largely confined in cereals to organs involved in plant reproductive processes has been an important advance. In barley, efficient plant regeneration was then achieved through the use of immature embryos (Luehrs and Loerz 1987), shoot apical meristems (Zhang *et al*. 1999), populations of uni- or early bicellular pollen either still contained in anthers (Kuhlmann and Foroughi-Wehr 1989) or following isolation (Mordhorst *et al*. 1993), isolated ovules at the zygote or early embryo stage, or of isolated zygotes (Holm *et al*. 1995). In most of these systems, multiple plant regeneration is based on the formation of secondary embryos or embryo-like structures. Such embryos emerge either directly or via intermediate callus formation and can derive from both somatic (e.g. zygotes, immature embryos) and gametophytic (e.g. microspores) cells. The regenerable tissues generated *in vitro* do not only represent true callus or well-formed embryos, but also any kind of intermediates of both. In contrast, thus far, other potential sources such as immature inflorescences, mature seeds, or shoot base segments including the elongation zone of leaves along with axillary buds (Pasternak *et al*. 1999) do not appear to be adequate for genetic transformation in barley, albeit some of such systems have been successfully used as gene transfer targets in other cereals.

Contemporary methods of barley genetic transformation include the use of attenuated *Agrobacterium tumefaciens* strains that represent a naturally evolved and artificially optimized vehicle to integrate recombinant DNA into the nuclear genome of plants. Since monocotyledonous species do not belong to the typical natural hosts of this soil-borne bacterium and indeed appear to be recalcitrant to its mechanism of transformation, direct DNA-transfer was the preferred principle in most of the early attempts to generate transgenic barley. After publication of several reports on transient expression of recombinant DNA obtained using different techniques of DNA-transfer and various cellular targets (e.g. Junker *et al*. 1987; Toepfer *et al*. 1988; Lee *et al*. 1989; Mendel *et al*. 1989; Kuhlmann *et al*. 1991), Lazzeri *et al*. (1991) were the first to provide compelling evidence of stable transgene integration and expression in barley. They used polyethylene glycol (PEG)-induced DNA-uptake into protoplasts isolated from immature embryo-derived cell suspensions. However, only cell lines stably expressing the genes introduced, but no transgenic regenerants were obtained in this study. Later, Funatsuki *et al*. (1995) reported the production of transgenic barley plants using a similar protocol. To circumvent the disadvantages of cell suspensions which are prone to somaclonal variation and a continuous loss of morphogenic competence, some other studies included primary callus as a source of protoplasts to be used to introduce foreign DNA. Salmenkallio-Marttila *et al*. (1995) generated transgenic barley lines via DNA-transfer into microspore culture-derived protoplasts following electroporation. In another successful approach, plasmid DNA was transferred into protoplasts isolated from immature embryo-derived callus through PEG-induced perforation of the plasma membrane (Kihara *et al*. 1998). Employing PEG-mediated DNA uptake into protoplasts isolated directly from scutella of immature embryos, i.e. without prior callus formation, Nobre *et al*. (2000) also obtained transgenic barley lines. In a particularly remarkable direct DNA-transfer approach, Holm *et al*. (2000) used isolated barley zygotes as targets for microinjection. The highly efficient regeneration system (Holm *et al*. 1994) utilised in this study permitted the generation of a number of plants with recombinant DNA sequences integrated, even under non-selective conditions. However, possibly due to frequent vector truncations and/or rearrangements during the procedure, stable transgene expression was not observed in the plants obtained. Considering that none of the aforementioned methods has so far been successfully applied using any effector gene, their generally limited potential becomes obvious.

By contrast, the introduction of biolistic gene transfer, i.e. the bombardment of target cells with accelerated, DNA-coated metal particles, represents a crucial step towards the establishment of robust transformation technologies for barley. Based on this method, immature embryos (Wan and Lemaux 1994; Cho *et al*. 1998; Harwood *et al*. 2000), isolated microspores rendered competent to undergo androgenetic development (Jaehne *et al*. 1994) and shoot meristematic cultures (Zhang *et al*. 1999) have been successfully used as gene transfer targets to generate stable transgenic barley. The latter approach has the particular advantage that the target cultures are obtained from germinated grains fol-

Table 1 Barley genotypes amenable to the generation of stable transgenic plants

Genotype	Method and target of gene transfer	Efficiency[a]	Reference[b]
cv. 'Golden Promise'	Biolistics, pre-cultured immature embryos (IEs)	7.9 plants/ 100 IEs	Wan and Lemaux 1994
	Biolistics, microspore-derived embryos	0.3 plants/ 100 explants	Wan and Lemaux 1994
	Agrobacterium mediated, pre-cultured IEs	7 plants/ 100 IEs	Tingay *et al*. 1997
	Agrobacterium-mediated, IEs	12 plants/ 100 IEs, 66% co-integration of marker-free T-DNA	Matthews *et al*. 2001
	Agrobacterium-mediated, pre-cultured IEs	9.2 plants/ 100 IEs	Murray *et al*. 2004
	Agrobacterium-mediated, isolated ovules	3.1 plants/ 100 ovules, without selection: 0.8 plants/ 100 ovules	Holme *et al*. 2006
	Agrobacterium-mediated, IEs	17.9 plants/ 100 IEs, 34.6% co-integration of marker–free T-DNA	Coronado *et al*. 2005
	Agrobacterium-mediated, IEs	86.7 plants/ 100 IEs	Hensel *et al*. 2007
cv. 'Igri'	Biolistics, isolated microspores following induction of androgenetic competence	1 plant/ 10^7 microspores	Jaehne *et al*. 1994
	PEG-induced, protoplasts from IE-derived cell suspensions	Very low	Funatsuki *et al*. 1995
	Microinjection, isolated zygotes	No stable expression	Holm *et al*. 2000
	Biolistics, androgenetically competent microspores	1 plant/ >$3x10^6$ microspores	Carlson *et al*. 2001
	Agrobacterium-mediated, androgenetic pollen cultures	2.2 plants/ spike	Kumlehn *et al*. 2006
cv. 'Kymppi'	Biolistics, IEs	< 1 plant/ 100 IEs	Ritala *et al*. 1994
cv. 'Galena'	Biolistics, pre-cultured IEs	Very low	Cho *et al*. 1998
cv. 'Harrington'	Biolistics, pre-cultured IEs	Very low	Cho *et al*. 1998; Zhang *et al*. 1999
	Biolistics, shoot meristematic cultures	0.8 plants/ 100 explants	
cv. 'Clipper'	PEG-induced, scutellum-derived protoplast	Very low	Nobre *et al*. 2000
cv. 'Schooner'	*Agrobacterium*-mediated, pre-cultured IEs	0.9 plants/ 100 IEs	Wang *et al*. 2001
cv. 'Conlon'	Biolistics, pre-cultured IEs	3.2 plants/ 100 IEs	Manoharan and Dahleen 2002
cv. 'Sloop'	*Agrobacterium*-mediated, pre-cultured IEs	0.6 plants/ 100 IEs	Murray *et al*. 2004
cv. 'Chebec'	*Agrobacterium*-mediated, pre-cultured IEs	0.6 plants/ 100 IEs	Murray *et al*. 2004
cv. 'Tafeno'	*Agrobacterium*-mediated, pre-cultured IEs	2 plants/ 100 IEs	Hensel *et al*. 2007
cv. 'Helium'	*Agrobacterium*-mediated, pre-cultured IEs	2 plants/ 100 IEs	Hensel *et al*. 2007
	Agrobacterium-mediated, IEs	0.3 plants/ 100 IEs	
PF17048-51	*Agrobacterium*-mediated, pre-cultured IEs	7 plants/ 100 IEs	Hensel *et al*. 2007
	Agrobacterium-mediated, IEs	8 plants/ 100 IEs	
PF18147-52	*Agrobacterium*-mediated, IEs	0.3 plants/ 100 IEs	Hensel *et al*. 2007
W122/37.1	*Agrobacterium*-mediated, IEs	3.2 plants/ 100 IEs	Hensel *et al*. 2007
cv. 'Optic'	*Agrobacterium*-mediated, IEs	0.5 plants/ 100 IEs	Hensel *et al*. 2007

[a]) The efficiencies obtained under the best conditions tested are given. Since genetically identical clones are typically included, the data may not represent the true efficiency of independent transgenic plant formation.

[b]) Note that only studies are listed in which convincing evidence for stable transgene integration was provided, and studies which did not result in any improvement of an earlier published method are also excluded.

lowed by meristem proliferation *in vitro*, so there is no need to provide adult donor plants of appropriate quality which is one of the major limits in all other methods of stable barley transformation. Unfortunately, there is as yet no report on the application of this technique which might be due to the comparatively low efficiency in transgenic plant formation achieved (**Table 1**).

Agrobacterium-mediated transformation of cereals has been considered a particular challenge and a multitude of initial attempts to employ this principle failed or have lead to false-positive results, as was stated by Potrykus (1990) and experimentally demonstrated by Langridge *et al*. (1992). The pioneering work of Hiei *et al*. (1994) in rice however, represents a breakthrough in cereal transformation in that it provided unequivocal evidence for the generation of a high number of independent plants with T-DNA stably integrated in the nuclear genome and the transgenes expressed. This success appeared to stimulate a new round in the race to develop *Agrobacterium*-based transformation protocols for monocotyledonous crop species including barley. Tingay *et al*. (1997) were then the first to present stable transgenic barley plants reproducibly obtained through co-cultivation of immature embryos with *A. tumefaciens.* From today's point of view, the success of this study was achieved thanks to the concerted effects of several particular conditions brought about by the experimental setup, e.g. the choice of the hypervirulent *Agrobacterium* strain AGL-1 along with an appropriate binary vector, growth of inoculum without addition of antibiotics, the use of the highly amenable cv. 'Golden Promise', the removal of the embryo axis, as well as an hitherto unusual orientation of the embryos with the scutellum placed in contact with the medium during and following co-cultivation. Later, Matthews *et al*. (2001) simplified and improved the method published by Tingay and co-workers, whereby immature embryos were directly co-cultivated with agrobacteria without prior culture on callus induction medium and without wounding. Murray *et al*. (2004), Hensel *et al*. (2007) and others (**Table 1**) eventually succeeded in the stable transformation of a number of further barley genotypes, albeit the frequency of transgenic plant formation turned out to be substantially higher in cv. 'Golden Promise' than in the other lines shown to be amenable to the transformation protocols used. In cv. 'Golden Promise', transformation efficiencies higher than 10 independent primary transgenic plants per 100 inoculated embryos have been routinely achieved by several groups (Matthews *et al*. 2001; Coronado *et al*. 2005; Hensel *et al*. 2007).

Although the use of immature embryos results in by far most efficient generation of transgenic barley, other cells and tissues with particular advantages were also successfully utilised for *Agrobacterium*-mediated transformation (**Table 1**). Isolated microspores represent very attractive targets for gene transfer, since millions of such immature pollen grains can be obtained from just one donor plant from which several hundred plants can be regenerated. However, the major potential advantage of this cell culture system is that microspores, in contrast to somatic cells, are haploid, thus providing the unique opportunity to immediately obtain transgenic plants that are homozygous for the introduced sequence. This true-breeding status would be brought about by either spontaneous or artificially induced genome doubling after the transgene integration event.

While earlier attempts to achieve *Agrobacterium*-mediated gene transfer to isolated microspores or to microspore-derived callus did succeed only in the generation of trans-

genic callus lines (Wu *et al*. 1998), Kumlehn *et al*. (2006) recently published a reproducible method of barley transformation based upon infection of androgenetic pollen cultures with agrobacteria. By specifically aiming to support the transformation activity of the agrobacteria while limiting their growth during as well as following co-culture, the optimised conditions established in this study resulted in adequate efficiency of transgenic plant formation. Haploid target cells provide the potential advantage to obtain homozygous transgenic plants one generation earlier than with somatic cell systems. However, for the immediate identification of such true-breeding primary transgenic lines it would be necessary that no hemizygous plants appear in parallel, because homozygous and hemizygous lines cannot be unambiguously distinguished from each other without analysing their progeny. Although for some of the resultant primary transgenic lines produced by Kumlehn *et al*. (2006) homozygosity of the transgene was shown, these lines still required discrimination from the more abundantly obtained hemizygous plants through copy number assessment along with time-consuming segregation analyses of the respective T_1-populations. However, for the first time, this unique method did not only result in the instant formation of homozygous transgenic plants, but also permitted the generation of transgenic barley of winter-type (cv. 'Igri'), as well as proved to be successful using non-hypervirulent agrobacteria (GV3101), albeit the transformation efficiency was substantially lower than with a hypervirulent derivative of LBA4404 (Komari *et al*. 1996).

Pursuing another novel approach, Holme *et al*. (2006) generated transgenic barley via infection of isolated ovules with *A. tumefaciens*. The frequencies of transgenic plant formation were fairly comparable with methods of barley transformation routinely used by others. Although stable transgene integration appeared to happen preferentially after the zygote stage, the transformed target cells did not undergo extensive dedifferentiation, so the resultant transgenic plants bear a comparatively low risk of being spontaneously mutated through somaclonal variation. However, the major potential advantages of this unique transformation method are that the ovule culture system utilised to regenerate plants from the genetically transformed zygotes or embryo cells is substantially less genotype-dependent compared with any other barley cellular target so far known to be amenable to transformation, and the method was proven to be effective at still reasonable efficiency without the use of selective conditions during the *in vitro* culture procedure.

In summary, there are three especially useful ways to generate stable transgenic barley. The gene transfer methods targeting androgenetic pollen cultures or isolated ovules have unique advantages regarding particular quality issues of the plants obtained. It is furthermore remarkable that these two systems are not available in any other species than barley, including the dicotyledonous species which are typically more amenable to *Agrobacterium*-based transformation than the monocotyledons such as cereals. Thanks to its incomparably high efficiency, the use of immature embryos as target for *Agrobacterium*-mediated gene transfer is the most widely employed barley transformation system. Consequently, it is presumed that each of these three methods will likely to be the subject of further optimisation and employment in future approaches for stable barley transformation.

Agrobacterium strains and binary vectors

To date, reproducible and efficient generation of transgenic barley plants has been confined to the employment of *A. tumefaciens* strains carrying an accessory set of virulence gene copies comprising *VirB*, *VirC1* and *VirG*, which render the bacteria hypervirulent (Komari *et al*. 1996). The *A. tumefaciens* strains AGL-0 and AGL-1, which are widely used for gene transfer to immature barley embryos (Tingay *et al.* 1997; Matthews *et al.* 2001; Murray *et al.* 2004; Coronado *et al*. 2005; Lange *et al*. 2006; Hensel *et al*. 2007), harbour these additional alleles on their Ti-plasmid-derived attenuated helper plasmid pTiBo542ΔT-DNA. Strain LBA4404 has been successfully used for gene transfer to both immature embryos (Coronado *et al*. 2005; Hensel *et al*. 2007) and androgenetic pollen cultures (Kumlehn *et al*. 2006). The LBA4404-derivative used in these studies contains the plasmid pSB1, which also carries the above accessory virulence gene copies contained on a 15 kb fragment of pTiBo542. In addition, pSB1 includes sites for the integration of a T-DNA of choice via homologous recombination with the intermediate shuttle vector pSB11 (Komari *et al*. 1996). However, Kumlehn *et al*. (2006) reported that LBA4404 (pSB1) along with a conventional binary vector resulted in higher reproducibility and efficiency of gene transfer to androgenetic pollen cultures compared with the co-integrative plasmid version. In the same article, Kumlehn and coworkers give the hitherto only example of transgenic barley plant formation based on the use of a non-hypervirulent *Agrobacterium* strain (GV3101::pMP90). However, the transformation efficiency has been significantly lower compared with the use of LBA 4404 containing pSB1.

Stable barley transformation has been achieved using many different binary vectors containing the various gene expression cassettes to be transferred. Regarding their backbones however, the variation of these vectors is rather little. Among the backbone elements, the agrobacterial origin of replication (ORI) is most critical for the maintenance and abundance of a given binary vector inside the agrobacteria. To the best of our knowledge, the ORIs used in binary vectors for barley transformation have so far been confined to pVS1, pRK2 or pSP72.

Selectable marker and reporter genes

Since preferential or even exclusive development of transgenic cells, tissues or plants is necessary to establish and employ powerful transformation systems, a selectable marker gene is typically linked and co-introduced with the gene of interest to render the transgenic cells resistant to a respective selective pressure. In many early attempts to transform barley, kanamycin resistance conferred by the *E. coli* neomycin phosphotransferase (NPT) gene (e.g. Lazzeri *et al*. 1991; Funatsuki *et al*. 1995) was used. In later studies however, it turned out that the selection systems based on the phosphinothricin acetyltransferase (PAT, BAR) genes derived from *Streptomyces* species (e.g. Wan and Lemaux 1994; Jaehne *et al*. 1994; Tingay *et al*. 1997) as well as on the *E. coli* hygromycin phosphotransferase (HPT) gene (e.g. Hagio *et al*. 1995; Manoharan and Dahleen 2002) are more effective in barley. Interestingly, the studies that have so far lead to the most valuable and the most efficient protocols are all based upon the use of hygromycin, irrespective of the diverse gene transfer targets and the respective regeneration systems employed (Matthews *et al*. 2001; Murray *et al*. 2004; Coronado *et al*. 2005; Holme *et al*. 2006; Kumlehn *et al*. 2006; Hensel *et al*. 2007). Reed *et al*. (2001) reported the successful introduction of an alternative selectable marker gene into barley, namely a phosphomannose isomerase (PMI) gene. PMI converts mannose-6-phosphate, which cannot be utilised for carbon assimilation by most plant species, into fructose-6-phosphate. Therefore, cells expressing the PMI gene can preferentially develop on medium containing mannose as carbon source. This selection system was reported to result in an efficiency of three transgenic barley plants per 100 inoculated immature embryos. However, detailed information neither on the experimental setup nor on the molecular evidence of transgene integration has yet been shown for this interesting approach.

Reporter genes are commonly used to display gene expression in transgenic cells, tissues and plants. In barley, the β-glucuronidase reporter gene (*uidA*, *gus*) has been the most widely utilised system. Although it is a destructive technique leading to the death of the plant material analysed, it

has been of great value for the establishment of barley transformation technologies, as is documented in a multitude of publications. Harwood *et al*. (2002) reported on the use of the firefly (*Photinus pyralis*) luciferase reporter gene (*luc*) for barley transformation. Following biolistic gene transfer to immature embryos, about 14% of the embryo-derived calli expressed the *luc* gene, but only one to two transgenic plants were obtained per 100 embryos bombarded. This system did not find further application in transgenic barley, presumably due to the fairly expensive equipment needed and the development of more suitable alternatives. In more recent studies, the green fluorescent protein (GFP) gene has been increasingly employed (Carlson *et al*. 2001; Fang *et al*. 2002; Murray *et al*. 2004; Holme *et al*. 2006; Kumlehn *et al*. 2006), as this reporter gene has several advantages compared with other reporter systems. For instance, it allows for the detection of transgenic cells without the cellular functions and plant development being necessarily compromised, it is a small molecule encoded by a accordingly small, easy-to-handle nucleotide sequence, there are derivatives available with different excitation and emission spectra, and it can be translationally fused with the coding sequences of proteins to be analysed. This does not only facilitate the intracellular localization of respective gene product (Deshmukh *et al*. 2006), but also allows to reveal interactions with other tagged proteins via techniques based upon fluorescence resonance (Shen *et al.* 2007).

Generation of transgenic barley free of unnecessary DNA-sequences

Selectable markers typically become useless once transgenic plants are produced, or even may prevent the use of the same marker gene for sequential transformation of the same line. Moreover, the presence of selectable marker genes has raised concerns because of the resultant resistance of crops to antibiotics or herbicides.

Several strategies have been developed to obtain transgenic plants which are free of selectable markers or of other unwanted recombinant DNA-sequences (reviewed by Hohn *et al*. 2001). The independent integration of unlinked DNA-sequences coding for the selectable marker gene and the gene of interest, respectively, followed by segregation of the two uncoupled integration loci in the progeny represents the most common way to get rid of selectable marker genes. To this end, Matthews *et al*. (2001) and Stahl *et al*. (2002) constructed twin binary vectors that carry two T-DNAs per binary vector to be used for barley transformation. The co-transformation efficiency in the primary transgenic plants obtained by Matthews and co-workers was 66%. Twenty-four per cent of these co-transgenic lines showed independent segregation of effector and marker genes in the T_1. This resulted in an overall segregation efficiency of 16% with regard to the primary transgenic plants initially generated. A disadvantage of this method is however, that it is relatively time consuming and laborious.

Coronado *et al*. (2005) developed a more rapid method that includes the co-transformation using a mixture of two *Agrobacterium* strains, one carrying the gene of interest and the other the selectable marker gene. The co-transgenic lines obtained with an efficiency of 34.6% were used for androgenetic pollen cultures. In the resultant segregating populations of doubled haploid plants, selectable marker-free transgenic lines were identified. A particular advantage of this approach is that these lines are not only marker-free, but also generally true-breeding for the transgene at a time.

In another approach, a conditional negative selection system for barley was developed by Koprek *et al*. (1999), where a naturally non-toxic substrate exerts a phytotoxic effect in cells that harbour the selectable marker gene *codA* whereas non-transgenic cells are not compromised. Following generation of primary transgenic plants by co-introduction of a conventional selectable marker linked to a *codA* expression cassette along with an unlinked T-DNA, this system is useful to obtain exclusive formation of selectable marker-free T_1-segregants.

There are some transformation systems which allow for genetic transformation without use of any selectable marker system. The only such example for barley has been recently reported by Holme *et al.* (2006). They were able to generate 0.8 stable transgenic barley plants per 100 isolated ovules inoculated with agrobacteria without selective conditions being applied during the entire process of plant formation.

Patterns of recombinant DNA integration and transgene expression

Stahl *et al.* (2002) were the first to publish a detailed analysis of patterns and sites of T-DNA integration in transgenic barley. This study revealed that the T-DNA integrates into the barley genome in a fairly comparable way as was earlier observed in dicotyledonous plants. Integration of multiple T-DNA copies into the same single locus occurred with a frequency of about 50%. All such repeats were of tandem configuration, i.e. no head-to-head or tail-to-tail orientation was encountered. About one third of the T-DNA/ plant DNA border junctions analysed regarding their chromosomal integration sites were found to be inserted into actively transcribed *BARE copia*-like retrotransposons. This

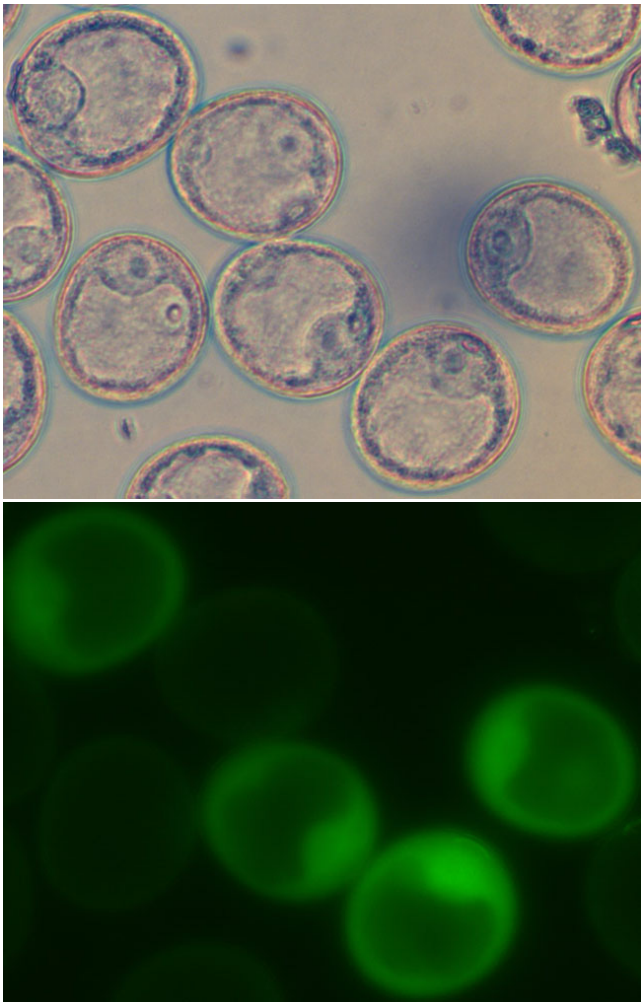

Fig. 1 Immature pollen from a primary transgenic barley plant of breeding line PF17048-51 carrying a single T-DNA copy according to Southern blot. The plant was generated by *Agrobacterium*-mediated gene transfer to immature embryos using a maize ubiquitin promoter::*gfp* construct (upper: under white light; lower: the same object exposed to far blue light and recorded with GFP-filter set). Considering the hemizygous state of the plant, about half of the pollen grains are expected to express the transgene. This is in accord with what can be seen on the lower picture.

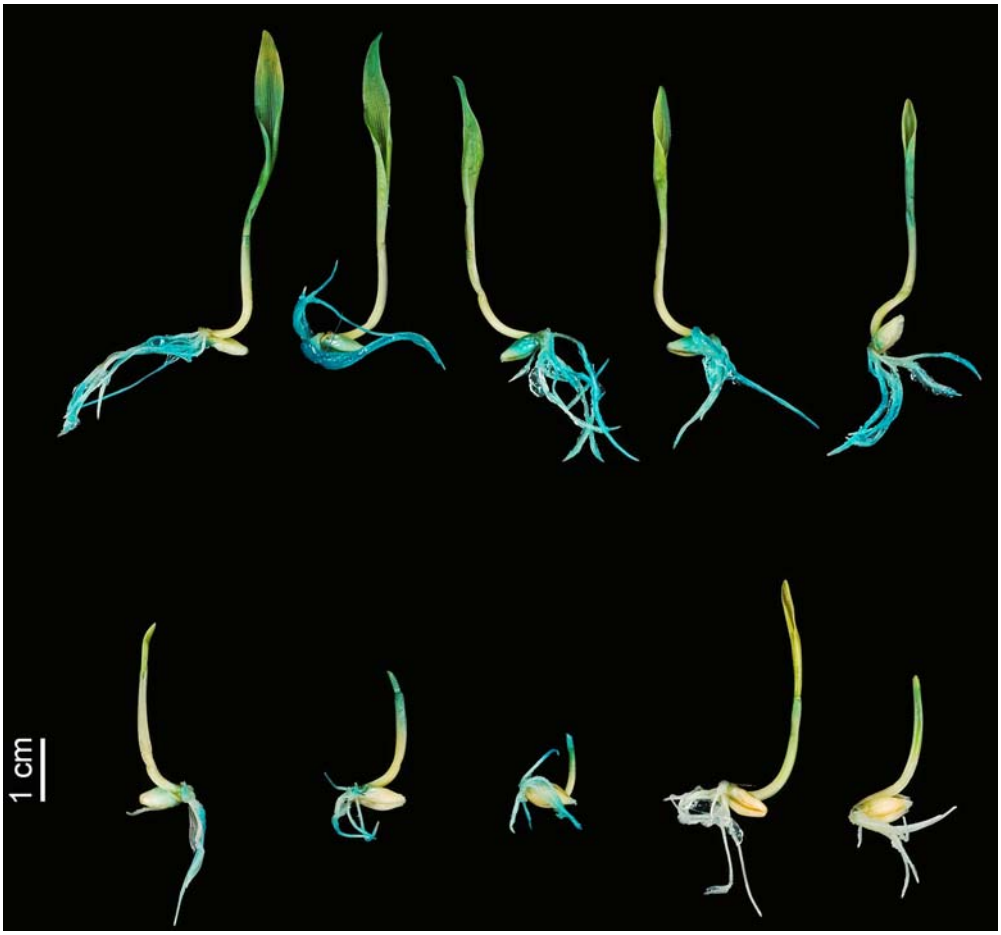

Fig. 2 Progeny of a primary transgenic barley plant of cv. 'Golden Promise'. The T_0-plant was generated by *Agrobacterium*-mediated gene transfer to immature embryos using a maize ubiquitin promoter::*gus* construct, and carried a single T-DNA copy according to Southern blot. The progeny plantlets were infiltrated with staining buffer containing X-gluc. A faint background colourisation is often observed in non-transgenic barley tissues as well. *Gus*-expression is recognised best in roots, while in leaves, the blue colour is largely masked by chlorophyll. Considering the hemizygous state of their mother plant, a three-to-one segregation of transgenic and non-transgenic T_1-plants is expected. The two plantlets at the lower right appear to be non-transgenic segregants.

finding was not surprising, as this transposon sequence is highly repetitive within the barley genome. Another study of T-DNA/ plant DNA border junctions revealed that the right T-DNA ends appeared to be highly conserved, whereas the left T-DNA ends were found to be more variable (Fang *et al.* 2002). Sequencing of four right and five left border junctions further showed that the backbone of the binary vector was always excluded from the sequence integrated into the barley genome. Salvo-Garrido *et al.* (2004) conducted physical and genetic mapping of DNA integration sites in transgenic barley lines obtained by particle bombardment or *Agrobacterium* infection of immature embryos. Using fluorescence *in situ* hybridisation, a total of 23 transgene integration sites were detected in five of the seven barley chromosomes, while no case of DNA integration was detected in the remaining two chromosomes. The integration patterns showed a rather non-random distribution and transgene integration did fairly coincide with the gene rich telomeric and subtelomeric regions. Another study which included transgenic barley generated using both agrobacteria and particle bombardment was performed by Travella *et al*. (2005). This comparative approach showed that *Agrobacterium* integrated between one and three copies of the T-DNA per individual transgenic plant, while only minimal rearrangements were found. In turn, 60% of the transgenic barley lines obtained via particle bombardment had more than eight copies of the transgene along with extensive DNA rearrangements.

Agrobacterium-mediated gene transfer to immature barley embryos conducted by Lange *et al*. (2006) resulted in 52% single copy transgenic lines, while 33% had two to three copies, and 15% possessed four insertions. In contrast to the above mentioned paper of Fang *et al*. (2002), Southern blotting with a vector backbone probe further revealed that about half of the analysed lines possessed vector backbone fragments. The results of Lange and co-workers regarding the transgene copy numbers are fairly comparable with what was recently published by Hensel *et al*. (2007) who obtained 50% single copy plants, 31% with two, 10% with three und 9% carrying more than three copies (for transgene segregation in pollen and in T_1 see **Figs. 1** and **2**, respectively).

The integration patterns of transgenic barley plants generated through *Agrobacterium*-mediated transformation of isolated barley ovules were analysed by Holme *et al*. (2006). About 37% of the lines contained a single T-DNA copy, 42% two copies, 10% three copies and another 10% had four copies. Some of the multiple transgene copies turned out to be physically linked, as was shown by segregation ratios in the respective T_1 populations.

For barley, there is thus far no conclusive data on gene silencing available. However, there is no doubt that it frequently occurs and largely follows the rules known from other plant species. This is basically supported by two observations. First, the relatively high copy numbers obtained in direct gene transfer methods coincide with a comparatively high proportion of transgenics that do not express the gene(s) introduced (Travella *et al*. 2005), and second, a few examples of gene silencing generated by the use of the RNAi approach (see below) imply that the general machinery of gene silencing does largely operate in barley as it has been shown in other plants.

RETRIEVAL OF DNA-SEQUENCES USED FOR TRANSGENIC APPROACHES

The worldwide available and constantly expanding barley genetic resources, along with the sophisticated tools for their detailed analysis and processing, represent highly useful sources for basic and applied research (**Table 2**).

In the major dicotyledonous crop species and in rice, insertion mutagenesis has been used extensively to create representative populations of knock-out mutant lines which are highly valuable for the tagging and the functional characterisation of the respective genes interrupted (Bouchez and Hoefte 1998; Martienssen 1998). The establishment of

Table 2 Representative overview on barley genetic resources and tools as major sources for the retrieval of DNA-sequences to be introduced into barley.

Resource, Tool	Feature	Reference
Information on barley genomic DNA		
Molecular markers and quantitative trait loci (QTLs)	Relative positions of genetic loci	http://rye.pw.usda.gov/cgi-bin/cmap/species_info
Genetic map	Overview on the relative positions of genetic loci within the genome	www.ncbi.nlm.nih.gov/mapview/map_search.cgi?chr=barley.inf; Stein *et al.* 2007
BAC library of cv. 'Morex'	6.3 genome equivalents, average insert size 106 kb	Yu *et al.* 2000
Expressed sequence tags (ESTs)		
	438,000 worldwide	www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html; Zhang *et al.* 2004
Databases and sequence processing tools		
Nucleotide and amino acid sequence data	Blast searches	www.ncbi.nlm.nih.gov/mapview/static/MVPlantBlast.shtml?3
CR-EST	Access to sequence, classification, clustering and annotation data	http://pgrc.ipk-gatersleben.de/cr-est; Kuenne *et al*. 2005
BARLEYBASE	Data visualisation and statistical analysis of plant microarrays	www.barleybase.org; Shen *et al*. 2005
GRAMENE	Comparative genome analysis in grasses	www.gramene.org; Jaiswal *et al*. 2006
PHRED	Generation of high-quality ESTs	www.phrap.org; Ewing and Green 1998
CAP3	EST alignment and clustering, identification of tentative singletons	http://deepc2.zool.iastate.edu/aat/cap/cap.html Huang and Madan 1999
Comprehensive cDNA-arrays		
Affymetrix 22K Barley1 GeneChip	22,792 probe sets derived from 84 cDNA libraries, represents at least 21,439 unigenes	Close *et al*. 2004
10K IPK array	10K cDNA array derived from 200,000 ESTs	Maraschin *et al.* 2006; Gjetting *et al.* 2007
Dedicated cDNA arrays		
	Germination	Potokina *et al.* 2002, 2004
	Maternal and filial tissues of caryopses	Sreenivasulu *et al.* 2002, 2004
	Leaves non-infected and infected by *Blumeria graminis* f.sp. *hordei*	Jansen *et al.* 2005; Eichmann *et al.* 2006

such populations can be achieved by the random integration of T-DNAs via *Agrobacterium*-mediated transformation, or by the introduction and spreading of appropriately designed transposable elements. The latter approach has been pursued by Koprek *et al*. (2000) using the maize Ac/Ds system in barley.

The utilisation of the various resources and tools (e.g. for the search for homologs to known genes, for comparative transcriptome analyses, or for positional cloning approaches) has resulted in the identification of DNA-sequences for which a detailed functional analysis and validation via transient and/or stable transformation of barley has been conducted. In turn, the potential of the ever-growing body of genetic resources and sequence processing tools, which is still far from being entirely exploited, has strongly stimulated efforts to develop and further improve powerful transformation technologies. In addition to what is found among the barley genetic resources, regulatory and coding nucleotide sequences from organisms other than barley are sometimes more useful to be transferred to barley, especially for the implementation of application-oriented genetic engineering strategies.

PROMOTER SEQUENCES USED TO DRIVE EXPRESSION OF TRANSGENES

The identification and employment of appropriate promoter sequences to drive expression of transgenes is vital to both the development of transformation technologies and its application in fundamental research and biotechnology. While promoters providing strong ubiquitous expression have been commonly used along with selectable marker and reporter genes in the scope of studies aiming to establish transformation methods, in recent years, it became increasingly desirable to have cell-, tissue or organ specific, or even inducible expression systems at one's disposal. Unfortunately, the promoters originating from dicotyledonous plant species, which cover a wide range of specificities and expression strengths do often not work appropriately, if at all, in the genetic context of monocotyledons. Therefore, the choice of promoters available for barley (and other cereals) is still fairly limited. An overview of the ubiquitous and specific promoters that have been used so far in transgenic barley is shown in **Table 3**. Because expression of recombinant proteins in mature or germinating grains may well represent the most promising biotechnological strategy in barley, it is not surprising that most of the promoters characterised to date are more or less strictly endosperm-specific (for an example see **Fig. 3**).

Table 3 Characterisation of promoter specificities via stable transformation of barley with promoter-reporter gene constructs.

Promoter	Specificity	Reference
Maize ubiquitin 1	Ubiquitous	Christensen and Quail 1996; Furtado and Henry 2005
CaMV 35S	Ubiquitous	Furtado and Henry 2005
Rice Actin1	Ubiquitous	Vickers *et al*. 2006
Barley Hor B1	Endosperm	Cho *et al*. 1999a, 2002; Vickers *et al*. 2006
Barley Hor D	Endosperm	Cho *et al*. 1999a, 2002
Oat Glo1	Endosperm	Vickers *et al*. 2006
Rice GluB-1	Endosperm	Patel *et al*. 2000
Barley Jekyll	Nucellar projection	Radchuk *et al*. 2006
Barley Asi	Embryo	Furtado *et al*. 2003
Barley Em	Embryo	Furtado and Henry 2005
Barley Lem1	Lemma/palea	Skadsen *et al*. 2002
Barley Lem2	Lemma/palea	Tilahun *et al*. 2006

FUNCTIONAL CHARACTERISATION OF GENES AND GENETIC ENGINEERING TOWARDS CROP IMPROVEMENT AND MOLECULAR FARMING

Unlike in rice and corn, more advanced transformation techniques for barley have only more recently become available. Therefore it is not surprising that approaches to functionally characterise candidate regulatory and coding DNA-sequences and to genetically modify and improve barley via stable transformation are lagging behind. Besides this situation, some studies have been conducted in barley as well. For the time being, the transfer of simple single-gene traits from other cultivars or cereals seems to be the most straightforward and promising strategy to create novel successful varieties. **Tables 4**, **5** and **6** provide a comprehensive overview on recombinant genes which have so far been stably expressed in barley. The genes compiled in these tables are associated with plant-pathogen interactions (**Table 4**), developmental processes and product quality (**Table 5**) as well as

Fig. 3 Mature T_1-grains of barley cv. 'Igri' obtained from a primary transgenic plant that was generated via *Agrobacterium*-mediated gene transfer to androgenetic pollen cultures using a wheat α-gliadin promoter::*gfp* construct (upper: under white light; lower: the same object exposed to far blue light and recorded with GFP-filter set). Note that the promoter drives strong *gfp* expression in the endosperm, while the embryos do not show any fluorescence.

with molecular farming approaches (**Table 6**).

Plant-pathogen interaction

Pathogen attack is a major concern in barley and attempts have been made to address this issue by the use of transgenic plants. However, a functional analysis of genes implicated in the complex molecular mechanisms of plant-pathogen interactions is often required to better understand these processes prior to directed approaches of genetic engineering towards improved resistance to pathogens. A comprehensive overview of published studies based on stable barley transformation using genes involved in plant-pathogen interactions are compiled in **Table 4**.

The barley eukaryotic translation initiation factor 4E gene (*Eif4E*) was identified to be putatively associated with resistance to Bymoviruses via a positional cloning approach. Through stable transformation of the resistant cv. 'Igri', which carries a naturally occurring mutant *eif4E* allele, with *Eif4E* from a susceptible barley background, Stein *et al*. (2005) provided compelling evidence that this gene represents an essential host factor for Bymoviruses.

A number of barley genes modulated upon infection by diverse fungal pathogens has been identified through comparative transcriptomics approaches and verified by functional transient expression studies (Douchkov *et al*. 2005; Hein *et al*. 2005; Kogel and Langen 2005; Shen *et al*. 2007). In many of these studies, the fungus *Blumeria graminis* f. sp. *hordei* has been established as an excellent experimental model for detailed examinations of molecular mechanisms that underlay barley-fungus interactions. Employing this model, a number of candidate barley genes implicated in such processes have been further studied via stable over-expression or knock-down. Stable over-expression of constitutively activated RACB, for instance, rendered barley more susceptible to powdery mildew (Schultheiss *et al*. 2005).

Based upon available information on useful gene functions, more directed approaches to genetic engineering have additionally been published in recent years. According to the major limits of conventional barley breeding strategies, numerous studies have aimed at the improvement of resistance to viral and fungal pathogens. The Barley Yellow Dwarf Virus (BYDV) causes a major viral disease. The expression of the BYDV coat protein gene under the control of the CaMV 35S promoter in barley resulted in plants that display a resistant phenotype (McGrath *et al*. 1997). In these experiments however, the level of resistance was inconsistent in the progeny. No determination of the abundance of the recombinant coat protein was made, so it is unclear whether sufficient expression was achieved using the 35S promoter and whether transgene expression did correlate with resistance. Wang *et al*. (2000) reported that the expression of a hairpin RNAi construct derived from a BYDV genomic sequence in barley resulted in a resistant phenotype in T_0 plants that properly segregated in the T_1 generation. This resistance was specific for BYDV and did not prevent an infection with Cereal Yellow Dwarf Virus. Whether this RNAi-mediated resistance will hold true in the field has not yet been tested.

The stem rust resistance gene *Rpg1* from the resistant

Table 4 Stable transformation of barley using genes involved in plant-pathogen interactions.

Coding sequence	Promoter, specificity	Effect	Reference
Interaction with viral pathogens			
BYDV-PAV coat protein gene	CaMV 35S, ubiquitous	BYDV resistance	McGrath *et al*. 1997
BYDV-PAV orf1/orf2-RNAi	Maize ubiquitin 1, ubiquitous	BYDV resistance	Wang *et al*. 2000
Barley eucaryotic translation initiation factor 4E Eif4E	Maize ubiquitin 1, ubiquitous	Susceptibility to Bymoviruses	Stein *et al*. 2005
Interaction with fungal pathogens			
Vitis vinifera stilbene synthase 1	*Vitis vinifera* stilbene synthase 1 with 4x CaMV 35S enhancer	Fungal resistance	Leckband and Loerz 1998
Barley stem rust resistance Rpg1	Barley Rpg1, ubiquitous	Stem rust resistance	Horvath *et al*. 2003
Barley mildew-resistance locus A mla1, mla6 (epitope-tagged)	Barley 2 kb of its native 5′ sequence	Fungal resistance	Bieri *et al*. 2004
Barley small RAC/ROP GTPase gene racB-G15V	Maize ubiquitin 1, ubiquitous	Susceptibility to powdery mildew and abiotic stress	Schultheiss *et al*. 2005
Barley BAX inhibitor-1 gene gfp-fusion gfp:BI-1	CaMV 35S, ubiquitous	Reduced fungal growth in root tissue	Deshmukh *et al*. 2006
Barley small RAC/ROP GTPase genes rac3-G17V, rac1	Maize ubiquitin 1, ubiquitous	Susceptibility to powdery mildew	Pathuri *et al.* unpublished

Table 5 Stable transformation of barley using genes associated with developmental processes and product quality.

Coding sequence	Promoter, specificity	Effect	Reference
Barley transcription factor Gamyb	Barley 1.5 kb native Gamyb 5′ sequence, aleurone and anther specific	Male sterility, increased expression of hydrolytic enzymes in aleurone	Murray *et al*. 2003; Murray *et al*. 2006
Barley phosphate transporter Pht1-6	Rice actin, ubiquitous	Altered phosphate uptake	Rae *et al*. 2004
Chimaeric sequence from *Bacillus amyloliquefaciens* and *B. macerans* thermotolerant (1,3-1,4)-β-glucanases[a]	Barley high-pI α-amylase, aleurone-specific	Improved feed and malt quality	Jensen *et al*. 1996, 1998; Horvath *et al*. 2000; von Wettstein *et al*. 2000, 2003
Escherichia coli aspartate kinase and dihydrodipicolinate synthase	CaMV 35S, ubiquitous	Change in soluble amino acids in leaf and grain	Brinch-Pedersen *et al*. 1996
Endo-β-1,4-xylanase[a], arabinofuranosidase[a], thermostable α-amylase[a], saccharifying amylase[a], endoprotease[a], exopeptidase[a], (1,3-1,4)-β-glucanase[a] from different microbial sources	Rice GluB-1, endosperm-specific	Improved brewing process	Souppe and Beudeker 1998
Trichoderma reesei thermotolerant endo-1,4-β-glucanase[a] Egi1	Barley high-pI α-amylase, aleurone-specific	Improved malt quality	Nuutila *et al*. 1999
Barley Jekyll	Barley Jekyll, nucellar projection-specific	Essential function for grain development	Radchuk *et al*. 2006
Wheat thioredoxin h[a] Tatrxh	Barley Hor B1 and Hor D, endosperm-specific	Improved malt quality	Cho *et al*. 1999b; Horvath *et al*. 2000
Neocallimastix patriciarum endo-β-1,4-xylanase xynA	Rice GluB-1, endosperm-specific, barley Hor B1, endosperm-specific	Improved feed quality	Patel *et al*. 2000
Modified barley thermotolerant β-amylase[a]	Barley β-amylase, endosperm-specific	Improved malt quality	Kihara *et al*. 2000
Low-pI α-amylase[a], α-glucosidase[a], high-pI α-amylase[a]	Barley high-pI α-amylase, aleurone-specific	Improved malt quality	Matthews *et al*. 2001
Maize γ-zein	Wheat Glu-1 D-2, endosperm-specific	Altered grain texture	Zhang *et al*. 2003
Chimaeric sequence from *Neocallimastix patriciarum* CelA and *Piromyces sp*. Cel6G 1,4-β-glucanases	Rice GluB-1, endosperm-specific	Improved feed and malt quality	Xue *et al*. 2003
Barley limit dextrinase inhibitor Ldi	Maize ubiquitin 1, ubiquitous	Modulated starch composition	Stahl *et al*. 2004
Barley Jekyll-RNAi	Barley Jekyll, nucellar projection-specific	Altered grain filling	Radchuk *et al*. 2006
Wheat malate transporter gene Almt1	Maize ubiquitin 1, ubiquitous	Aluminium tolerance	Delhaize *et al.* 2004

[a]) Construct contained a signal peptide for targeting the recombinant protein to the endoplasmic reticulum

cv. 'Morex' was expressed in cv. 'Golden Promise' in order to transfer resistance to this susceptible genotype (Horvath *et al.* 2003). Traditional crossing and backcrossing can also achieve a transfer of a resistance gene between barley cultivars, but this generally requires much more time. As respective resistance genes do not exist for all fungal pathogens, other strategies have been discussed, namely the expression of anti-fungal proteins and peptides (Dahleen *et al.* 2001). Such an approach was reported by Leckband and Loerz (1998). They expressed the stilbene synthase 1 gene from grapevine, which should result in the accumulation of the phytoalexin resveratrol. Barley plants transgenic for the stilbene synthase gene showed reduced disease symptoms in a detached leaf assay after inoculation with *Botrytis cinerea*. The expression of enzymes capable of detoxifying fungal toxins should be a way to reduce toxin levels, and in the case these toxins are pathogenicity factors this strategy may also reduce disease symptoms. Manoharan *et al.* (2006) reported the expression of the *Tri101* gene from *Fusarium sporotrichioides*, encoding a 3-OH trichothecene acetyltransferase that can detoxify deoxynivalenol (DON), a protein synthesis inhibitor and the major mycotoxin in Fusarium head blight (FHB). Greenhouse-grown barley plants transgenic for the *Tri101* gene displayed a reduced FHB infection and a reduced DON level when compared to the non-transgenic control. However the authors did not find significant differences neither in the FHB infection nor in the DON level in plants tested during field trials. The authors argue that DON is not a pathogenicity factor in barley and that the harsher and more variable conditions in the field might overrun any effect *Tri101* expression might have on FHB.

Product quality

Most published studies on application-oriented transgenic approaches in barley were examining product quality improvement as feed, malt or as brewing ingredient. A comprehensive overview on the use of transgenic barley to study and to modulate grain development is included in **Table 5**.

In order to increase the digestibility of barley grains in monogastric animals like poultry, enzymes capable of digesting β-(1,3-1,4)-glucans were expressed in developing or germinating grains (Jensen *et al.* 1996, 1998; Nuutila *et al.* 1999; Horvath *et al*. 2000; Patel *et al.* 2000; Xue *et al.* 2003). It turned out that the expression of the transgene products in protein bodies of the developing endosperm by the use of storage protein gene promoters from cereals resulted not only in adequate expression levels, but also superseded the need for malting (Horvath *et al.* 2000; von Wettstein *et al.* 2003). Von Wettstein and coworkers (2003) reported that only 0.2 g of transgenic barley grains expressing 0.7 mg glucanase per gram grain is sufficient to supply enough enzyme activity into the intestinal tract of chickens to assure that 620 g of barley are digested properly, thereby increasing the nutritive value of barley to that of corn.

Enzymes that degrade starch, endosperm cell wall materials and proteins are important for the brewing process. Expression of the respective endogenous barley genes is induced during germination, which is part of the malting process. In order to enhance the malt quality through further support of these processes, over-expression of such barley genes or ectopic expression of bacterial or fungal genes with appropriate function was achieved during germination by several research groups (Jensen *et al.* 1998; Nuutila *et al.* 1999; Horvath *et al.* 2000; Kihara *et al.* 2000; Patel *et al.* 2000; Xue *et al.* 2003). A high activity in β-glucanases and β-xylanases is also thought to decrease viscosity of the wort and so enhances filterability. In the case where an ectopically expressed gene encodes an enzyme more thermo-tolerant than the endogenous barley enzyme, higher enzyme activities can be obtained after the drying of the barley germlings at elevated temperatures. An increased activity of starch debranching enzyme was found in germinating barley

Table 6 Overview on approaches to use barley as molecular farming system.

Coding sequence	Promoter, specificity	Effect	Reference
Enterotoxigenic *Escherichia coli* fimbrial adhesin FaeG[a]	Barley trypsin inhibitor promoter, endosperm specific	Grains containing edible vaccine to be feeded to piglets	Joensuu *et al*. 2006
Human antithrombin III[a], α_1-antitrypsin[a], serum albumin[a], lysozyme[a], lactoferrin[a]	Barley High-pI α-amylase promoter, aleurone specific, and barley Hor B1 promoter, endosperm specific	Molecular farming of pharmaceutical proteins	Stahl *et al*. 2002
Synthetic anti glycophorin scFv-HIV epitope fusion[a]	Wheat HMW glutenin *Bx17* promoter, endosperm specific	Molecular farming of HIV diagnostic reagent	Schuenmann *et al*. 2002
Human lysozyme[a]	Rice GluB-1 promoter, endosperm specific	Molecular farming of pharmaceutical proteins	Huang *et al*. 2006

[a]) Construct contained a signal peptide for targeting the recombinant protein to the endoplasmic reticulum

expressing wheat thioredoxin *h* (Cho *et al.* 1999b). The combined use of different transgenic barley grains ectopically expressing seven different malting enzymes in the endosperm have been shown to reduce the necessary amount of malt in the brewing process: Souppe and Beudeker (1998) showed that a replacement of about three-quarters of the barley malt by unmalted barley from which 10% was a mixture of the seven transgenic lines, resulted in a beer of similar taste than a control brew.

Molecular farming

Compared to other cereal grains, winter barley has a relatively high protein content (12-15%). This along with the high versatility of barley in terms of potential growing conditions as well as the low producer and storage costs for cereal grains make the use of barley caryopses as bioreactor for molecular farming a very interesting choice. Furthermore, the strictly self-pollinating nature of barley as well as its incapability of producing fertile hybrids with other related crops or wild species provides an inherent mechanism that practically prevents unwanted spreading of genetically modified germplasm under field conditions. Therefore, it is no surprise that companies like ORF Genetics (Reykjavik, Iceland) and Maltagen Forschung GmbH (Andernach, Germany) that are active in the field of molecular farming, have chosen barley as their production system. Barley is competing with other such systems mainly in the producer costs, which in turn strongly depend on the amount of recombinant protein produced per kg grain. A high abundance of the recombinant protein is thus central to successful molecular farming. Reported expression levels of pharmaceutical proteins in barley range from 0.15 g/kg grain for a single chain variable fragment antibody (scFv)-HIV epitope fusion (Schuenmann *et al.* 2002) to 3.0 g/kg grain for human serum albumin (HSA) or the sweetener protein thaumatin (Maltagen Forschung GmbH, http://www.maltagen.de, http://www.thaumatin.de). The expression level of foreign proteins in barley endosperm could be increased by optimizing the codon usage of the respective genes to fit the barley preference of G and C in the third base position (Jensen *et al.* 1996; Horvath *et al.* 2000; Xue *et al.* 2003) or by targeting the recombinant protein to the protein bodies by adding an N-terminal signal peptide (Cho *et al.* 1999a; Horvath *et al.* 2000).

Areas in molecular farming for which applicable examples in barley are developed are the production of pharmaceutical proteins like growth factors and cytokines (ORF Genetics, http://www.orfgenetics.com), oral vaccines (Joensuu *et al.* 2006; Maltagen Forschung GmbH, http://www.maltagen.de), a diagnostic reagent (Schuenmann *et al.* 2002) and food additives (Maltagen Forschung GmbH, http://www.maltagen.de). **Table 6** summarises the approaches to molecular farming by the use of barley as expression system.

Field performance

Very little research has been done on field performance of transgenic barley. Horvath *et al.* (2001) tested transgenic barley cv. 'Golden Promise', expressing a β-(1,3-1,4)-glucanase, over a period of three years in field trials. They found that the expression of the transgene remained constant over time and that the α-amylase promoter used to drive the expression of the β-(1,3-1,4)-glucanase turned leaky, resulting in expression also in the embryo of the mature grain. They also reported a reduced 1000-grain weight and a reduced yield compared to the untransformed cultivar. The authors speculated that this might be due to somaclonal variations induced by tissue culture during the transformation procedure. The occurrence of a reduced fitness in field grown transgenic barley was also reported by Manoharan *et al.* (2006), who were also discussing somaclonal variations as the cause. They backcrossed transgenic lines with the original wild type (wt) cv. 'Conlon', which in turn led to plants carrying the transgene and having a wt-like growth habit. The crossing of transgenic lines to elite cultivars by Horvath *et al*. (2001) resulted in homozygous lines that express the transgene and give a higher 1000-grain weight and yield than the untransformed cultivar, demonstrating that it is possible to enhance the agronomic features of transgenic barley by cross-breeding with elite cultivars.

PERSPECTIVES

The technologies that are now at our disposal to perform stable barley transformation will tremendously facilitate the efficient and comprehensive analysis of gene function. This will enhance our understanding of biological mechanisms and will help to complement barley breeding programmes by genetic engineering. The transformation procedure itself does not anymore constitute the major obstacle for such projects, as was the case in the past for quite some time. Nevertheless, a better understanding of the cellular particularities of the transformation process and further technical improvements would be highly beneficial for future applications. Furthermore, substantial advances in the respective regulatory framework and in the public perception of plant genetic engineering, especially in Europe, are required to enable biotech companies, plant breeders, farmers and consumers to substantially benefit from genetically modified plants with their particular advantages.

Despite the huge amount of genetic resources that are available, the lack of genomic sequences substantially limits basic and applied research in barley and its relatives. However, this situation may at least partially be resolved in the near future. Current activities to develop a sufficiently detailed physical map of the barley genome will provide a major technical prerequisite for following large-scale sequencing programmes. The International Barley Genome Sequencing Consortium (IBSC) has been constituted recently. It will focus on efforts to gain financial support for concerted activities that ultimately will lead to the sequencing of the barley genome (http://barleygenome.org). Along with more comprehensive genomic sequence information, the generation of barley TILLING and T-DNA insertion mutant populations may greatly facilitate the identification and cloning of genes implicated in any biological mechanism of interest.

To further increase the throughput of barley transformation, it would be highly desirable to provide generic vectors that allow for a comfortable integration of any kind of

DNA-sequence to be expressed. A more versatile set of vectors would also comprise vector derivatives with appropriate integration sites for diverse selectable marker gene cassettes as well as for different promoters to drive either constitutive, various cell- and tissue-specific or inducible expression of transgenes. Other vectors have to be generated that allow the integration of a given coding sequence adjacent to a screenable marker or an affinity tag that may later facilitate the cellular localisation of the resultant chimaeric gene product or its purification by providing particular binding properties, respectively. In fact, all those visions are not new; however to the best of our knowledge, no such comprehensive vector system is so far available for monocotyledonous plants. Here the major actual limitation is that promoters derived from dicotyledons are typically not useful to drive gene expression in barley and other cereals. In addition, only relatively few promoters derived from *Poaceae* species have been isolated and functionally characterised. So far also missing are chemically inducible expression systems that reliably operate in this taxon. Such inducible expression systems would permit the functional characterisation and biotechnological utilisation of genes that severely compromise plant development upon ubiquitous or tissue-specific expression.

Tremendous progress has been made in the elucidation of the general mechanisms of *Agrobacterium*-mediated gene transfer to plant cells including the recruitment of host factors (reviewed by Tzfira and Citovsky 2006). Moreover, it has been demonstrated that even non-plant eukaryotic organisms including yeast, fungi and human cells *in vitro* can be transformed by *A. tumefaciens* (reviewed by Lacroix *et al*. 2006). However, experimentally validated and detailed information about optimal conditions and particular requirements of agrobacteria for their efficient attachment and T-DNA transfer to target cells is still fairly limited. The current state of our understanding of these biological mechanisms may be sufficient for practical applications in most dicotyledonous plant species, yet the development and improvement of transformation technology for atypical *Agrobacterium* hosts such as barley will likely require a broader base of consolidated knowledge. In particular, comprehensive experiments on the physical and chemical conditions and their implications in *Agrobacterium*-mediated gene transfer may result in exceedingly valuable information. A number of appropriate barley transformation protocols as well as methods to quantify the success of gene transfer are available for such investigations.

The elucidation of particularities that limit the amenability of cells from monocotyledonous plants as target for *A. tumefaciens* would be of prime importance to facilitate the development of gene transfer protocols for genotypes or for regenerable cell types that are beyond the range of current barley transformation methods. For example, a routinely applicable and efficient transformation method based on the use of mature barley seeds or of target cells present shortly after their germination would render the tedious and time-consuming production of adult donor plants dispensible.

The targeted introduction of foreign DNA to known chromosomal destination sites constitutes another technical challenge. Such method may entirely rule out unforeseeable positional effects including unwanted disruption of endogenous genes through integration of recombinant DNA.

The immediate formation of homozygous transgenic barley plants may be achieved by gene transfer into haploid target cells such as egg cells or microspores followed by plant regeneration and spontaneous or chemically induced genome doubling. An especially realistic approach includes the identification of haploid primary transgenics that are frequently obtained following *Agrobacterium*-mediated gene transfer to androgenetic pollen cultures (Kumlehn *et al*. 2006). These selected haploid transgenics can be subjected to colchicine treatment that is thought to result in entirely true-breeding transgenic T_1-families that would not require confirmation through segregation analysis. This latter approach will most likely be accomplished in barley rather than in any other plant species, because an adequate cell culture and transformation system along with all other necessary technical prerequisites does already exist.

Also, gene transfer methods that are independent of agrobacteria are of interest and have to be improved. Broothearts *et al*. (2005) showed that gene transfer to plant species including rice can be achieved using suitably modified bacteria other than *A. tumefaciens* and *A. rhizogenes*. So far no such attempts were made in barley. This approach would be of particular value in application-oriented projects where freedom-to-operate is desired. Furthermore, biolistic transformation is still the method of choice in the field of plastid transformation, which represents one of the most promising strategies to achieve high-level expression of genes encoding valuable recombinant proteins to be produced in transgenic plants at a large scale.

As barley is thought to be well suited for molecular farming, the development of adequate grain-specific expression systems represents one of the actual technical challenges for plant biotechnologists. Improvements seem to be possible not only regarding the recombinant gene expression level, but also concerning post-translational modifications, targeted trafficking of recombinant cargo to cellular storage compartments and efficient deposition therein.

In the years to come, the transformation technologies already developed for barley will drive applied research on topics such as feed and malt quality, resistance to pathogens and to harsh environmental conditions, as well as high-level expression of valuable recombinant protein in barley grains. Despite all current achievements there is a multitude of opportunities to further advance barley transformation technology in the context of the co-evolution of fundamental research, biotechnology and practical plant breeding.

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