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REVIEW

Genetic transformation of barley: limiting factors

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Abstract

This review summarizes main difficulties involved in barley (*Hordeum vulgare* L.) transformation. The most commonly used procedures for genetic transformation in barley are *Agrobacterium tumefaciens* and particle bombardment mediated methods. While different barley cultivars are used for genetic engineering with varying sensitivity, recent improvements in regeneration and transformation techniques are described and summarized. Furthermore, some of the transformation complicating factors, in particular somaclonal variation and transgene insertion sites, are discussed in more detail.

Additional key words: *Agrobacterium*, albinism, *Hordeum*, particle bombardment, somaclonal variation, transgene insertion site.

Introduction

Genetic transformation of crop species is a tool for improving agronomic traits. Monocotyledonous species include a great number of agriculturally important crops such as maize, wheat, rice and barley. Barley (*Hordeum vulgare* L.) is utilized for its malting quality in brewing and distilling, and for its nutrition as animal feed or human aliment.

Microprojectile bombardment was the first successful method for barley transformation (Ritala *et al.* 1994, Wan and Lemaux 1994). Different tissues were used for microprojectile DNA transfer - immature embryos, callus derived from immature embryos, microspores and shoot meristematic cultures (Hagio *et al.* 1995, Jähne *et al*. 1994, Ritala *et al.* 1994, Wan and Lemaux 1994, Zhang *et al.* 1999). Holm *et al.* (2000) transformed barley by microinjection of DNA into zygote protoplasts, but transgene expression was rarely achieved. Fertile transgenic barley was also obtained by direct gene transfer to protoplasts – *via* polyethylenglycol-mediated uptake or electroporation (Funatsuki *et al.* 1995, Salmenkallio-Marttila *et al*. 1995, Kihara *et al.* 1998). However, a disadvantage of this method is the time-consuming initiation of embryogenic cell suspension cultures. The statement that *Agrobacterium* is not possible to use for transformation of monocotyledons (Potrykus 1990) has been refuted after the achievements with cereals (Chan *et al.* 1992, Hiei *et al.* 1994, Cheng *et al.* 1997, Ishida *et al.* 1996). Successful transformation of barley by *Agrobacterium tumefaciens* was first reported by Tingay *et al.* (1997). The efficiency of T-DNA delivery to cereal cells was improved by using the super-virulent *Agrobacterium* strain AGL1. The

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Abbreviations: AA - amino acid; Act1 - actin 1; Adh1 - alcohol dehydrogenase 1; AG - *Agrobacterium*-mediated transformation; BAP - benzylaminopurine; BYDV - barley yellow dwarf virus; CKX - cytokinin dehydrogenase; 2,4-D - 2,4-dichlorophenoxyacetic acid; DON - deoxynivalenol; *gfp* - gene for green fluorescent protein; GUS - β-glucuronidase; IEs - immature embryos; IPK - inositol-phosphate kinase; *luc* - luciferase gene; LTR - long terminal repeat; MEC - modified embryogenic callus; PB - particle bombardment; QTL - quantitative trait locus; SEC - standard embryogenic callus; SMC - shoot meristematic culture; Ubi1 - ubiquitin 1; UTR - untranslated region; ZFN - zinc-finger nuclease.
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modified transformation procedure used by Tingay *et al.* (1997) consisted of wounding immature embryos without embryonic axis by shooting the scutellum surface with 1 μm gold particles and subsequent inoculation with the AGL1 strain. The bombardment prior to co-cultivation induced a wound response. Acetosyringone is now used as an agent causing chemically induced wound response. However, Bartlett *et al.* (2008) did not confirm the effectiveness of acetosyringone used during co-cultivation to achieve high transformation efficiencies.

A substantial advantage of *Agrobacterium*-mediated transformation (Karami *et al.* 2009) compared to microprojectile bombardment is the possibility to transfer large segments of DNA with only minimal rearrangement (Hiei *et al*. 1997, Shibata and Liu 2000). Other advantages include low copy number integration, higher transformation efficiency and a higher percentage of stable T-DNA inherited as a simple Mendelian trait. On the contrary, transgene silencing and rearrangements have been frequently observed in transformants produced by direct DNA delivery (Travella *et al.* 2005). In Travella´s study, all barley lines produced by *Agrobacterium* transformation integrated between one and three copies of the transgenes, with minimal rearrangements, whereas 60 % of the barley lines transformed by particle bombardment integrated more than eight copies of the transgenes, with many rearrangements. Multi-copy insertion of a transgene has often been associated with gene silencing in transgenic plants produced by direct DNA delivery (Pawlowski and Somers 1996). However, single-copy and single-locus transformants also undergo silencing (Stoger *et al.* 1998, Kohli *et al.* 1999).

The main goal of barley transformation is improvement of its quality traits by expression of new genes. The transformation procedure was already utilized in research focusing on improving characteristics such as malting quality (Wang *et al.* 2000, 2001, Manoharan *et al.* 2006), disease resistance (Nuutila *et al.* 1999, Kihara *et al.* 2000, Tull *et al.* 2003), amino acid composition (Hansen *et al.* 2007, Lange *et al.* 2007), and quality of grains used as feed (Xue *et al.* 2003). Transgenic barley tolerating high levels of some toxic chemicals were also examined in a polluted environment (Kim *et al.* 2003, Delhaize *et al.* 2004). Interestingly, barley seeds were chosen as a bioreactor for molecular farming (Schünmann *et al.* 2002, Joensuu *et al.* 2006). Companies like *ORF Genetics* (Reykjavik, Iceland) and *Maltagen Forschung* (Andernach, Germany) have started to produce pharmaceutical proteins (growth factors, cytokines, oral vaccines, food additives) in transgenic barley lines with robust endosperm-specific expression.

In recent years, a considerable progress in barley transformation has been achieved. This review summarizes particular improvements during transformation procedures which have led to increasing transformation efficiency and successful regeneration or transformation of different barley cultivars. Somaclonal variation and transgene insertion sites, which are in connection with transgene stability, are discussed in more detail.

Transformation efficiency

Most published barley transformation experiments showed low transformation efficiency (Table 1). An innovation in transformation procedures was the use of an intermediate step when calli on induction or regeneration media are exposed to low irradiance of 10 - 30 μmol m^{-2} s^{-1} (Cho *et al.* 1998), or 75 μmol m^{-2} s^{-1} (Bartlett *et al.* 2008) resulting in a reduction in the frequency of regenerated albino plants (Fig. 1). Copper is thought to be an important microelement. Media containing increased concentrations of copper (5 μM) improved callus quality and regenerability (Dahleen 1995). Other modifications in microelement concentrations included increased H_3BO_3 (0.75 mM) and decreased $FeSO_4$ (0.05 mM; Dahleen and Bregitzer 2002). The regeneration frequency was also increased by adjustment of NH_4^+ content in the regeneration medium. Wan and Lemaux (1994) used regeneration medium where the concentration of NH_4NO_3 was ten fold lower than that of MS medium (Murashige and Skoog 1962) and furthermore, glutamine was added. Nuutila *et al.* (2000) demonstrated that the higher concentrations of organic nitrogen are necessary during the early embryogenesis, whereas inorganic nitrogen in the form of nitrates is required for shoot development. Amino acids (glutamine, proline or casein hydrolysate) provide a source of reduced nitrogen. Synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methoxy-3,6-dichlorobenzoic acid (dicamba) are commonly used in barley callus-induction media. Also 4-amino-2,5,6-trichloropicolinic acid (picloram) was successfully used during cereal transformation (Przetakiewicz *et al.* 2004). Dicamba and 2,4-D promoted similar regeneration frequencies; however, dicamba was superior in supporting long-term regeneration ability in barley. In addition, dicamba was generally superior to 2,4-D in promoting transient expression and subsequent stable transformation (Trifonova *et al.* 2001). According to Castillo *et al.* (1998), dicamba was superior to 2,4-D and picloram in the induction and maintenance of embryogenesis and regeneration capacity. Another study showed that optimum ratio between 2,4-D and benzylaminopurine (BAP) helped to maintain the regeneration of green plantlets better than dicamba and BAP (Jiang *et al.* 1998). Addition of BAP increased the quality of calli and its regenerability and reduced the incidence of albinism (Cho *et al.* 1998). However, low cytokinin contents should be maintained for initial callus

Table 1. Efficiency of selected barley transformations (cv. Golden Promise). These data may not represent the true efficiency since genetically identical clones could be included. Efficiency is expressed as the number of plants per 100 transformed embryos/ovules. AA - amino acid, AG - *Agrobacterium*-mediated transformation, BYDV - barley yellow dwarf virus, IEs - immature embryos, PB - particle bombardment.

Method	Efficiency [%]	Selection gene	Promoter/Gene of interest	Goal	Reference
PB, IEs/ micro-spore-derived Es	7.9/0.3	Ubi1/*bar*, 35S Adh1 intron/*bar*	reporter gene: Ubi1, Adh1/*uidA* (35S/BYDV-PAV)	first transformation by PB	Wan and Lemaux 1994
AG, IEs	1.7 - 7	Ubi1/*bar*	reporter gene: Act1/*gus*	first *Agrobacterium*-mediated transformation	Tingay *et al.* 1997
PB, IEs	15.2	Ubi1/*bar*	hybrid high-pI α-amylase/ thermostable endo-(1,4)-β-glu-canase	improved malt quality	Nuutila *et al.* 1999
AG, IEs	13	35S/*hpt*	hairpin RNA construct: Ubi1/BYDV-PAV polymerase	resistence to BYDV (PAV strain)	Wang *et al.* 2000
AG, IEs	2 - 12	35S/*hph*	high-pI α-amylase/ α-amylase, α-glucosidase	marker gene elimination	Matthews *et al.* 2001
PB, IEs	1.4	Ubi1/*bar*	high-pI α-amylase/α-amylase (*alkBA*)	increased activity improved malt quality	Tull *et al.* 2003
AG, IEs	17.9	35S/*hpt*	reporter genes: Act1/*gus*, Ubi1/*gfp*	marker-free transgenic plants	Coronado *et al.* 2005
AG, IEs	4.4, 9.2	35S/*hph*	reporter genes: Ubi1/*gfp*, *gus*	comparison of reporter gene expression	Murray *et al.* 2004
PB, IEs	1	Ubi1/*bar*	trypsin inhibitor promoter/faeG	grains containing vaccine	Joensuu *et al.* 2006
AG, ovules	3.1	35S/*hpt*	reporter gene: Ubi1/*gfp*	ovule transformation	Holme *et al.* 2008
AG, IEs	5.4	35S/*hpt*	Ubi/antisense C-hordein encoding gene	more balanced AA composition	Lange *et al.* 2007
AG, IEs	25	35S/*hpt*	reporter gene: Ubi1/*luc*	improvement of transformation	Bartlett *et al.* 2008
AG, IEs	4 - 86.7	35S/*hpt*	reporter genes: Ubi1/*gfp*, Act1/*gus*	improvement of transformation	Hensel *et al.* 2008
AG, IEs	2.8	Ubi1/*bar*	Ubi1/*gus*	improvement of transformation	our unpublished data
AG, IEs	5 - 74	35S/*hpt*	Ubi1/*dapA*	increased content of free-lysine	our unpublished data

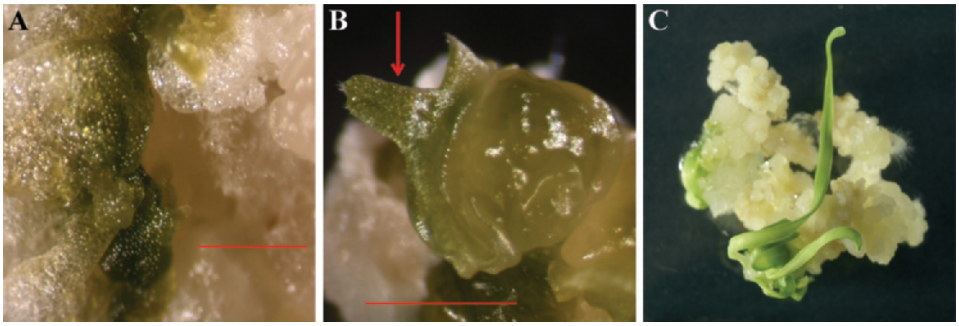

Fig. 1. Plant regeneration from embryogenic callus of barley cultivar Golden Promise: *A* - callus exposed to low irradiance (80 μmol m^{-2} s^{-1}), *bar* 1 mm; *B* - green regenerative structure (*arrow* shows developing shoot), *bar* 1 mm; *C* - regeneration of shoot under irradiance of 160 μmol m^{-2} s^{-1}.

induction because the high cytokinin content decreases the initial growth rate of the callus. Thus, callus initiation on medium with auxin alone and inclusion of an intermediate sub-culture medium containing auxin, cytokinin and copper before regeneration increased green shoot production (Cho *et al.* 1998). Our research was focused on transformation of barley with cytokinin dehydrogenase gene (*CKX*). For callus formation, after inoculation with *Agrobacterium,* we used medium containing 2,4-D and BAP. Since plant regeneration on hormone-free medium failed, probably due to transgene action, we regenerated plants on medium with 1 mg dm^{-3} of BAP. We also successfully used the specific inhibitor of CKX – 2-chloro-6-(3-methoxyphenyl)aminopurine

(Zatloukal *et al*. 2008), together with 2,4-D, for callus induction and maintenance on sub-culture medium. This inhibitor induced regeneration with efficiency comparable to BAP (unpublished data). Rooting of regenerated shoots was performed on medium without hormones.

Bartlett *et al.* (2008) developed a protocol with average transformation efficiencies of 25 %. Moreover, they reported that their recent experiments yielded transformation efficiencies over 50 %. Immature embryos were transformed with *Agrobacterium tumefaciens* (strain AGL1) harboring pBract vectors enabling selection on hygromycin. Copper at 5 μM was used in callus induction and transition sub-culture media. Regeneration medium did not contain additional copper (copper concentration was the same as in Murashige and Skoog medium). Dicamba (2.5 mg dm^{-3}) was used in the callus induction medium, 2,4-D (2.5 mg dm^{-3}) and benzylaminopurine (0.1 mg dm^{-3}) were added to the transition medium. The regeneration medium contained no hormones. All medium components, with exception of *Phytagel*, were filter sterilized to prevent the adverse effects of autoclaving. It is thought that filter-sterilized media give improved results, since autoclaving could induce unwanted interactions between medium components and the production of toxic products from sugar breakdown. It was also reported that high and stable transgene expression can be kept by incorporating of the intron at a specific position within the coding sequence (Bartlett *et al.* 2009). Hensel *et al.* (2008, 2009) established a transformation protocol that gave efficiency up to 86.7 stable transgenics per 100 immature embryos of cultivar Golden Promise. The co-cultivation medium was improved by adding 800 mg dm^{-3} of L-cysteine and 500 μM acetosyringone. Infected embryos showed no sign of necrosis or abortion, probably due to the anti-oxidative effect of L-cysteine.

The most important factor influencing high-throughput transformation is the availability of immature embryos from good quality plants grown under strictly controlled conditions. Plants should not be sprayed with fungicides or insecticides. Optimal growth conditions, especially appropriate watering, must be kept through the whole lifespan of embryo-donor plants. Transformation efficiency increased from 5 to 70 % (Table 1) when greenhouse-grown donor plants (cv. Golden Promise) were substituted with plants grown under optimum conditions (Bartlett *et al.* 2008). Another essential factor for obtaining a high-number of transformants is use of an appropriate selection agent. In our hands, hygromycin selection works much more effectively then selection on the herbicide bialaphos.

Barley cultivars

Most transformation protocols were developed for the model cultivar, Golden Promise, which is not agriculturally important. However, these protocols are not suitable for many commercially important barley cultivars, which have low plant regeneration frequencies (Bregitzer *et al*. 1998a) or callus-induction response rates (Jiang *et al.* 1998). Many studies have compared regeneration ability of different barley cultivars in relation to medium composition. The changes in the transformation protocol described by Cho *et al.* (1998) resulted in successful transformation of the North American barley cultivars Galena and Harrington.

Separate autoclaving of certain culture media components and reducing the amount of callus per Petri dish improved regeneration of the cultivars Harrington, Morex and Hector. Regeneration improvement in response to various concentrations of copper and 2,4-D were genotype specific (Bregitzer *et al.* 1998a). Castillo *et al*. (1998) studied the regeneration ability of different barley cultivars (18 of the two-row type and 14 of the six-row type). Effect of three different auxins was also compared in three selected cultivars. Dicamba was superior 2,4-D and Picloram for the induction and maintenance of callus. Dahleen and Bregitzer (2002) reported improved plant regeneration of cultivars Morex, Harrington, Foster, Drummond, Conlon, Colter, 90Ab321, Baronesse and Crystal by modifying iron and boric acid concentrations, and by adding BAP (0.1 mg dm^{-3}) to maintenance medium. The optimal size of embryos for callus formation on induction medium containing 3 mg dm^{-3} 2,4-D or dicamba and following production of green plants with fewer albinos was 0.5 - 1.5 mm in cv. Morex (Chang *et al*. 2003). Shoot regeneration was performed on medium with 0.5 - 1.0 mg dm^{-3} BAP and shoots were rooted with 0.2 mg dm^{-3} indole butyric acid in the medium. Twelve spring barley cultivars registered in the Czech Republic were tested for callus induction and regeneration capacity. Most cultivars created more regenerated plants after callus induction with 2,4-D compared to picloram or dicamba. Cultivars Atribut, Forum and Scarlett were selected as the most suitable cultivars for further study (Šerhantová *et al.* 2004). Jha *et al.* 2007 found that increased ethylene production, caused by addition of 1-aminocyclopropane 1-carboxylic acid, within weeks 8 to 10 of *in vitro* culturing enhanced the regeneration of cv. Morex. On the contrary, blocking of ethylene activity by silver nitrate during weeks 5 to 10 also increased regeneration of cv. Morex (almost 2-fold) and Golden Promise (1.5-fold). He and Jia (2008) developed an efficient plant regeneration system from mature embryos with endosperm of highland barley (*Hordeum vulgare* var. *nudum*). Successful formation of embryogenic calli from leaf base segments of seven semi-winter,

commercial cultivars from China and following regeneration was recently reported (Li *et al.* 2009).

Transgenic barley was obtained from cv. Harrington by bombardment of shoot meristematic cultures (SMCs) derived from germinated seedlings (Zhang *et al.* 1999). Advantages of SMCs are high plant regeneration and enhanced genomic stability in comparison to embryogenic callus. Shoot meristematic cells do not go through a callus or de-differentiation phase; vegetative shoots can be directly induced from shoot meristematic cultures. Manoharan and Dahleen (2002) successfully transformed the barley cultivar Conlon by bombardment of embryo-derived callus. Hensel *et al.* (2008) improved transformation conditions for the spring cultivars Helium, Optic, PF17048-51, PF18147-52, W122/37.1 and the winter cultivar Tafeno.

Australian cultivars Schooner, Sloop and Chebec were also successfully transformed by *Agrobacterium* (Wang *et al.* 2001, Murray *et al.* 2004). Roussy *et al.* (2001) tested five Nordic cultivars (Baronesse, Cecilia, Filippa, Mentor, Pongo) for transformation and regeneration capacity. Pongo, Baronesse and Filippa showed the best results. Holme *et al*. (2008) reported that plant regeneration from young barley embryos derived from *in vitro*-cultured ovules is genotype independent. Cultivars Femina, Salome, Corniche and Alexis, which are known to have poor response in other types of tissue cultures, were successfully transformed using the protocol for *Agrobacterium* infection of ovules.

Selection of transgenic homozygous plants

Barley is a self-pollinated plant. Foreign genes (transgenes) integrated into the barley genome are transmitted to progenies in a Mendelian manner. In practical breeding, homozygous plants with a stable transgene are required. The process used for identification of transgenic homozygous plants requires screening of a large number of plants in the T1 and T2 generations, which is laborious and time-consuming (Massiah *et al*. 2001). Homozygous transgenic plants of barley can be rapidly produced by androgenic segregation through anther culture (Müllerová *et al*. 2001), embryogenic pollen culture (Coronado *et al*. 2005) and microspore culture (Ritala *et al*. 2005). The anther culture technique in T1 and T2 progeny of transgenic barley cv. Golden Promise was successfully applied. The transgenic line HB1A (T2) was transformed with plasmid pAHC25 (Christensen and Quail 1996) containing both the *gus* and *bar* genes. Transgenic T1 lines HH3E and HH1A were co-transformed with plasmids pAL51 (Lonsdale *et al*. 1995) and pAMFIT (provided by Prof. C. Fogher). The androgenic response of three transgenic lines was 47 %, From a total of 4160 cultured anthers, 60 green and 270 albino plant regenerated. A larger portion (65 %) of haploid and tetrahaploid plants was obtained in the androgenic progeny of transgenic lines compared with control. The segregation ratio in the spontaneously dihaploid green plants was 1:1 (Müllerová *et al*. 2001). Production of double haploid transgenic plants through anther or microspore culture can be used for the fast creation of homozygous transgenic plants.

Kumlehn *et al.* (2006) used *Agrobacterium* infection of androgenetic pollen cultures for barley transformation. After infection with *Agrobacterium tumefaciens* strains LBA4404 and GV3101, about 31 and 69 %, respectively, of the primary transgenic plants carried a single copy of the transgene. Four out of 20 T1 lines did not segregate for the reporter gene. Thus, identification of homozygous plants is possible one generation earlier in comparison to conventional transformation procedures. Shim *et al.* (2009) reported improvements in transformation of isolated barley microspores. The best procedure included a 4 h pre-bombardment cultivation on high-osmotic medium (0.5 M mannitol plus sorbitol) at either 4 or 25 °C. Additionally, arabinogalactan protein was added to the microspore culture medium and the actin promoter was used.

Albinism

Albinism is a common problem during barley transformation (Kasha *et al.* 1990). Albinism can be influenced by genetic background (Foroughi-Wehr *et al.* 1982), physiological state of the donor plants (Goldstein and Kronstadt 1986), exposure to bialaphos (Wan and Lemaux 1994), time in culture (Bregitzer *et al.* 1995a) and culture conditions (Ziauddin and Kasha 1990, Kao *et al.* 1991). Wan and Lemaux (1994) also mentioned that ammonia released by non-transformed cells during selection (Tachibana *et al.* 1986) can contribute to the occurrence of albinism.

The suitable irradiance of calli early in the selection process reduced the incidence of albinism (Cho *et al.* 1998). The creation of green sectors on calli caused by exposure to sufficient irradiance ensures that green plants will be regenerated. Albinism is also linked to changes in plastid DNA that occur during re-differentiation (Mouritzen *et al.* 1994). Bregitzer and Campbell (2001) identified one quantitative trait locus (QTL) for green plant regeneration and at least one for albino plants. They

also confirmed previously reported associations of three QTLs with green plant regeneration (Komatsuda *et al*. 1995, Mano *et al*. 1996).

Somaclonal variation

Genetic changes, arising from *in vitro* culture, were termed somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variation is a serious problem during barley transformation. Structural rearrangements and variation in chromosome number can be found in plants from *in vitro* culture. For agronomic application, it is essential to improve a particular trait without changing the basic genetic background. Some factors are known to affect chromosomal instability in regenerated, nontransgenic plants. These include: plant species, genotype, initial ploidy level, explant source, medium composition, growth regulators, and time in culture (Constantin 1981). For example, polyploidy and aneuploidy in barley are positively correlated with increasing time in culture (Ziauddin and Kasha 1990, Wang *et al.* 1992).

Barley plants (cv. Golden Promise) derived from non-transgenic and transgenic callus obtained *via* microprojectile bombardment were compared regarding ploidy (Choi *et al.* 2000a). Variation in ploidy was greater in transgenic compared to nontransgenic plants: 54 % of the transgenic lines were diploid (2n = 2x = 14) and 46 % of the transgenic lines were tetraploid (2n = 4x = 28) or aneuploid around the tetraploid level, while only 0 - 4.3% of the nontransgenic plants were tetraploid. Ploidy changes in transgenic plants were more frequent, probably due to additional stresses during transformation. The DNA introduction process involved exposure of cells to vacuum, cellular damage due to microprojectiles, and potential loss of cell pressure potential following particle impact. Moreover, transformed tissues grow in the presence of dead tissue during selection. Choi *et al.* (2000a) also tested media used for culture initiation, *in vitro* culture conditions during selection and inclusion of osmotics into media (bombardment of osmotically treated embryos). Differences in the frequency of chromosomal aberrations were not observed. However, ploidy changes occur most probably early during the selection on bialaphos. Similar results were described by Bregitzer *et al.* (1998b). Based on our unpublished data, we confirm such observations since the transformants produced from particle bombardment of Golden Promise showed tetraploidy in high frequency when selected on bialaphos. Particle bombardment of immature embryos of the cv. Golden Promise induced great somaclonal variation during culture. One of the many potential mutagenic factors taken into account was the use of phosphinothricin, which resulted in a locally altered pH and increased ammonia levels (Bregitzer *et al*. 1998b). Choi *et al.* (2001) published that selection on bialaphos or hygromycin B and osmotic treatment (cultivation of embryos on medium with 0.2 M mannitol and 0.2 M sorbitol for 4 h before bombardment) cause extensive cytological aberrations in transgenic barley. Their investigation did not prove a significant effect of bombardment itself on frequency of aberrations in cells of calli. The damage of immature embryos caused by the particles was significantly less in cv. Golden Promise than in cv. Galena (Koprek *et al.* 1999).

Choi *et al.* (2000a) found that cells of callus tissue were aneuploid around the diploid chromosome number, but no non-transgenic and transgenic plants were aneuploid around the diploid number. However, high numbers of transgenic plants were aneuploid around the tetraploid level. The authors suggested that polyploid cells were probably more buffered to the effects of gene losses in aneuploids due to gene dosage. Other chromosomal changes, such as point mutations, small deletions or insertions or methylation polymorphism, were also observed (Choi *et al.* 2001).

Choi *et al.* (2000a) observed phenotypic variation related to ploidy. Tetraploid or near-tetraploid plants had delayed growth rates, broader leaves, thicker roots, spikes that did not emerge completely from leaf sheaths and seeds that were longer in comparison to those of diploid plants. Aneuploid plants (mostly with chromosome numbers of 26 or 27) had an abnormal grass-like phenotype. Their sterility was probably related to the instability of chromosome number during an abnormal meiosis. Morphological variation was also observed in 18 cultivars of barley lines derived from tissue culture (Ullrich *et al*. 1991). Height, heading date, spike length, spikelet density, grain yield, and malting quality were negatively affected in tissue-culture-derived plants of several barley cultivars (Bregitzer and Poulson 1995, Bregitzer *et al.* 1995b). Prostrate and spreading growth habit, excessive dwarfism, extremely slow development, several types of chlorophyll mutations, and premature death were observed in many transgenic lines of cv. Golden Promise (harboring genes *bar*, *uidA*, BYDVcp; Bregitzer *et al*. 1998b).

Cho *et al*. (1999) published that T0 plants from 6 of 12 independent transgenic lines were tetraploid and out of these 6 lines, plants from only one gave a ratio consistent with 35:1 segregation of GUS expression. This means that cells were diploid and became tetraploid after DNA integration. Plants from the rest of the lines gave a ratio consistent with a 3:1 segregation of expression. Thus, DNA integrations had already occurred in tetraploid cells (Choi *et al.* 2000a).

Bregitzer *et al*. (2002) tested three different tissue culture systems – standard embryogenic callus tissues

(SEC; Wan and Lemaux 1994), shoot meristematic cultures (SMC; Zhang *et al.* 1999), and modified embryogenic callus tissues (MEC; Cho *et al*. 1998). Agronomic traits (heading date, plant height, grain yield and quality) were measured for plants regenerated from cultures of two barley genotypes and compared with those from uncultured controls. SEC and MEC tissues were derived from scutellar tissues of immature embryos under different cultivating conditions and SMC tissues were derived from axillary meristems of *in vitro* germinated immature embryos. Long term culture capable of high levels of plant regeneration is an advantage of MEC and SMC. A disadvantage of SMC is slow initial development. The greatest reductions in agronomic performance were from plants derived from standard embryogenic callus tissues. On the contrary, plants derived from highly differentiated, meristematic tissues had the least reduction in agronomic performance. SEC-derived plants were generally shorter than MEC- and SMC-derived plants. SMC-derived plants had the highest yield and grain weight whereas SEC-derived plants had the lowest values. Moreover, MEC and SMC tissues showed minimal losses of plant regeneration (even in cultures older than one year). This increase in plant regeneration may reflect greater genomic stability. Nevertheless, Bregitzer *et al*. (2002) did not report that SMC tissues are superior to the MEC tissues with respect to somaclonal variation.

Bregitzer *et al*. (2008) recently published a new approach to reduce negative changes in transgenic barley. Single backcrosses of transgenic lines with wild-type were used for eliminating agronomic and quality alterations caused by somaclonal variation.

Transgene insertion sites – transgene stability

It is desirable to integrate a transgene into a position in the genome where transcription is active. The transgene insertion site influences transgene stability. However, it is still not possible to target a transgene into a particular site of a host genome since homologuous recombination is not routinely implemented in higher plant cells.

Transgene insertion into a host plant genome is thought to be a fully random process. A study focused on distribution of transgene insertion sites in barley revealed the presence of transgenes only on five (2H, 3H, 4H, 5H, and 6H) of the seven barley chromosomes (Salvo-Garrido *et al*. 2004). Fluorescent *in situ* hybridization was used for determination of the physical position of transgenes. Subsequent confirmation of the precise location of the transgenes was determined by genetic mapping. Most of the integration sites were found on chromosomes 5H (30 %), 4H (30 %), and 6H (22 %). Salvo-Garrido *et al.* (2004) detected the most transgene insertions in the telomeric and subtelomeric regions of both the long and the short arms (39 %). 17 % of insertions were in the centromeric regions. Other regions of the long arms contained 22 % of the insertions, and the short arms also contained 22 %. Moreover, specific regions were found on chromosomes 4H and 5H with clusters of transgene insertions (on the short arm of chromosome 4H, on both the long arm and the short arm of chromosome 5H) suggesting that these areas are probably more susceptible for transgene insertion than other regions. Genomic regions flanking the transgene were gene-rich areas. It is estimated that only 12 % of the barley genome contains coding sequences (Barakat *et al*. 1997). Therefore, it is evident that integration of transgenes is not a fully random process. These conclusions support studies in *Arabidopsis thaliana* and rice. Qin *et al.* (2003) observed "hot regions" in the *Arabidopsis* genome that contained more insertions than others and most of the insertion sites were located in, or close to, genes. Also, Barakat *et al.* (2000) confirmed the presence of transgenes in gene-rich regions of *Arabidopsis* and rice by localizing T-DNA in fractions of DNA separated according to their GC levels. Sha *et al.* (2004) examined T-DNA flanking regions in rice and they detected preferential insertion into the coding areas of the genome.

Choi *et al.* (2002) used fluorescence *in situ* hybridization for raw mapping of transgenes and screening for homozygous transgenic barley prepared by microprojectile bombardment. No preferential integration sites among the chromosomes were found; however, within a chromosome a distal preference for transgene integration was observed. Transgenes in distal and telomeric regions of the chromosomes were observed in 58 % of the transgenic lines. Other integration sites were in centromeric and subtelomeric regions, and in satellite regions of the chromosomes.

Not only the transgene locus but also stress can affect transgene stability. Plant transposable elements can be activated by stress, mainly during *in vitro* culture (Grandbastien 1998). These elements can be inserted into novel sites, and therefore can cause somaclonal variation. Meng *et al*. (2006) observed no effect of environmental stress on transgene expression stability or methylation status in multi- or single-copy transgenic lines; however, environmental stresses (water, nutrient deprivation and elevated temperature) caused numerous morphological changes. On the contrary, complete or partial heritable transgene silencing was observed after six generations of stable expression (*uidA* and *bar* gene driven by the maize *ubiquitin*-1 promoter) in one multi-copy subline following passages *in vitro*. Transcriptional gene silencing correlated with methylation in the 5´UTR (5´nontranslated exon) and intron of the *ubi1* promoter complex and condensation of chromatin around the

transgenes. The subline contained stable, transcription-competent, inverted repeats of the transgene and the 3´LTR (long terminal repeat) from a gypsy-like barley retrotransposon Sabrina-1. Meng *et al*. (2006) concluded that the transgene locus itself may affect its tendency to silence after *in vitro* culture and transgene silencing might result from host defense mechanisms activated by changes in plant development programming and/or stress imposed during *in vitro* growth.

Koprek *et al*. (2001) reported that transposon-mediated single-copy gene delivery increases transgene expression stability. Barley plants expressing the maize *Ac* (*activator*) transposase were crossed with plants containing one or more copies of the selection gene inserted between the inverted-repeat *Ds* (*dissociation*) ends. Transgene expression in F2 progeny with the transposed *Ds*-selection gene in different locations was 100 % stable, while stable expression in plants without activated transposition of the selection gene was only 23 %. Analysis of the integration site in single-copy plants showed that the transposed transgene was inserted into transcriptionally active regions of the genome, whereas the original location of the transgene was in redundant or highly repetitive genomic regions.

Perspectives

Plant genetic engineering is a powerful tool for the study of gene function, and for the realization of increased yields through plant breeding. Transformation technologies have been successfully utilized in barley improvement for the production of elite cultivars with desired characteristics such as high and stable yields, feed and malting quality, resistance to pathogens, stress tolerance and high-level expression of valuable recombinant proteins in barley grains (Dahleen and Manoharan 2007, Goedeke *et al.* 2007, Godwin *et al.* 2009).

Development of transformation protocols which would be effective with minimal negative aspects, such as albinism, somaclonal variation or transgene inactivation is a main goal of laboratories working on transformation of barley and other cereals. Many innovations have resulted in resolution of these problems and also increased the efficiency of transformation using agriculturally important cultivars. The recent work of Bartlett *et al.* (2008) showed a big increase in transformation efficiency of the model cv. Golden Promise. If their procedure is applicable for cultivars of agricultural importance, it will be beneficial for future biotechnology procedures. In the future, transgenic barley can play an important role in high-quality food and feed production and in various industrial applications.

A novel strategy for plant genetic manipulation was recently developed (Shukla *et al*. 2009, Townsend *et al*. 2009). Designed zinc-finger nucleases (ZFNs) induced a double-strand break at the target locus. ZFNs were used to modify endogenous loci in plants of maize and tobacco protoplasts. Shukla *et al.* (2009) reported that simultaneous expression of ZFNs and delivery of a simple heterologous donor molecule leads to precise targeted addition of a herbicide-tolerance gene at the intended locus. Modified maize plants transmit genetic changes to the next generation. Shukla *et al.* (2009) chose as a target the *IPK1* gene encoding inositol-phosphate kinase that catalyzes the final step in phytate biosynthesis in seeds. Insertional disruption of the target locus, *IPK1*, results in both herbicide tolerance and alteration of inositol phosphate profile in developing seeds. This new promising strategy can have relevance for precise genetic manipulation of other cereal plant species such as barley in future (*e.g*. targeted gene knock-out).

Various techniques are under development for the removal of selectable marker genes in barley transgenic lines (Matthews *et al*. 2001, Xue *et al.* 2003); as production of marker-free plants is necessary to public concerns over the safety of genetically engineered crops. Chloroplast genetic engineering offers several advantages over nuclear genetic engineering, including gene containment in non-transgenic pollen and enhanced gene expression. Chloroplast transformation technology could be developed for barley and used for production of valuable recombinant proteins, such as those which need to be produced in large quantities and those which have prokaryotic origin.

Our effort is focused on preparation of transgenic barley with altered cytokinin content and increased lysine content. Transgenic barley with increased activity of cytokinin dehydrogenase (CKX, enzyme responsible for cytokinin degradation) showed retarded growth of the shoots and enhanced proliferation of the root system. Hence, root-driven expression of CKX in barley is a promising genetic manipulation to improve agricultural important traits such as higher tolerance to drought and better nutrient uptake. Furthermore, silencing of CKX expression in the aleurone layer of the barley grain could result in the accumulation of active cytokinins leading to the activation of cell-wall invertases, key enzymes in regulation of nutrition flow from source to sink tissues (Roitsch and González 2004). Thus, the spatial increase of cytokinin content can strengthen sink activity of the grain and have a direct effect on yield. Genetic engineering is also useful for improving nutritional quality, such as contents of vitamins, essential amino acids and minerals. Wheat, barley and maize grains contain insufficient content of lysine. We prepared several lines of barley plants overexpressing dihydrodipicolinate synthase, the key enzyme in the biosynthesis of lysine and other essential amino acids. These plants are currently being tested for increased free-lysine content.

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