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Stability of transgene expression, field performance and recombination breeding of transformed barley lines

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Abstract Stable inheritance of the transgene, consistent expression and competitive agronomic properties of transgenic crops are important parameters for successful use of the latter. These properties have been analyzed with 18 homozygous transgenic barley lines of the cultivar Golden Promise. The lines originated from three independent primary transformants obtained by the biolistic method with three plasmids containing respectively, the *bar* gene, the *uidA* gene and the gene for a protein-engineered heat-stable (1,3–1,4)-β-glucanase. Three production levels of recombinant β-glucanase were identified in homozygous transgenic T_3 plants, and these remained constant over a 3-year period. In micro-malting experiments, the heat-stable enzyme reached levels of up to 1.4 $\mu g \cdot mg^{-1}$ protein and survived kiln drying at levels of 70–100%. In the field trials of 1997 and 1998 the transgenic lines had a reduced 1000-grain weight as well as variable yield depressions compared to the Golden Promise progenitor. In 1999 large-scale propagations of the lines with the highest recombinant enzyme synthesis during germination and of Golden Promise were studied at three different locations. In an irrigated field transgenic lines yielded approximately 6 $t \cdot ha^{-1}$ and Golden Promise 7.7 $t \cdot ha^{-1}$. Cross-breeding was carried out to transfer the transgene into a more suitable genetic background. Crosses of the semi-dwarf *ari-e* mutant Golden Promise gave rise to the four morphological phenotypes nutans, high erect, erect, and *ari-e*. Two improvements were achieved: (1) F_3 lines homozygous for the expression of heat-stable (1,3–1,4)-β-glucanase were found among lines that were homozygous for each of the four morphological phenotypes; (2) improved 1000-grain weights and yields with respect to those of the original transformants were observed in some F_4 lines homozygous for the morphological phenotypes and for the transgene. In the case of a homozygous nutans line, the transgenic plants had a higher 1000-grain weight than those lacking the transgene. Like mutants providing useful output traits, transgenic plants will often have to be improved by relocating the gene into more suitable genotypes.

Keywords *Hordeum vulgare* · Transformation · Agronomic characteristics · Glucanase · Malting

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Introduction

In a self-pollinating diploid crop like barley, variation evolved primarily by mutation and selection. Since the middle of the last century more or less pure lines in the form of land-races have been collected and crossed. This recombination breeding has been supplemented since 1927 with the isolation of induced mutations, first using ionizing radiations and subsequently chemical mutagens to increase the rate of spontaneous mutations. The establishment of genetic transformation using the biolistic method or the *Agrobacterium*-mediated procedure has added a further barley crop improvement method for breeding (Wan and Lemaux 1994; Tingay et al. 1997; for review Horvath et al. 2000).

Selection for improved grain weight, better grain yield, disease resistance and climatic adaptation through the selection of spontaneous mutations has shaped the modern barley crop plant as early as 5000 years ago. Further significant improvements in yield and quality characteristics have been achieved over the last century by recombination breeding and tedious genome fitting of

induced mutations (von Wettstein 1995). In the case of genetic transformation, useful or interesting genes are isolated from plants, animals or microorganisms and carefully characterized as to their function. In the transformation process, ideally, a single gene with its appropriate controlling elements is added to the genome instead of moving a whole chromosome or chromosome segment with many known and unknown genes as is done with the traditional hybridizations. One might expect that this procedure requires less genome fitting than an induced mutation or a gene introduced by hybridization. As yet we have no or only limited possibilities to target the transgene into a suitable chromosomal position by homologous recombination and, therefore, the insertion of the transgene may interfere with an endogenous gene and thus require the analysis of a large number of independent transformants in order to select a suitable candidate.

Bombardment of immature zygotic embryos of the malting barley cultivar Golden Promise with three plasmids gave rise to 14 fertile T_0 transgenic plants (Jensen et al. 1996). One plasmid contained the gene for a protein-engineered heat-stable (1,3–1,4)-β-glucanase driven by an aleurone-specific, high-pI α-amylase promoter. The two other plasmids carried the *uidA* gene for β-glucuronidase expression and the *bar* gene for expression of phosphinotricin transacetylase under the control of the constitutive promoters synthetic pEmu and maize pUbi-1, respectively. Mendelian inheritance of the heat-stable transgenic locus was demonstrated for progenies of the primary T_0 transformant 6.2.2. A copy number of six was estimated in heterozygous plants and of 12 in homozygous plants (Jensen et al. 1998).

In the project presented here we have analyzed 18 transgenic Golden Promise lines originating from three primary T_0 transformants (6.2.1, 6.2.2, 8.2.1) for the following, (1) stability of expression of the protein-engineered (1,3–1,4)-β-glucanase during germination with an aleurone-specific promoter and the β-glucuronidase (GUS) marker protein driven by the constitutive pEmu promoter, over three generations; (2) the agronomic characteristics of the transgenic lines, such as 1000-grain weight and yield, in 3 years of field trials; (3) the temporal synthesis of the recombinant heat-stable β-glucanase during malting; (4) optimization of grain quality and agronomic characteristics of the transgenic Golden Promise lines by cross-breeding the transformants to present elite cultivars.

Materials and methods

Field trials

Field trials were conducted with transgenic Golden Promise plants obtained by the biolistic method as previously described (Jensen et al. 1996). T_2 and T_3 generations of three T_0 plants expressing the heat-stable (1,3–1,4)-β-glucanase during germination were grown in a field nursery at the Spillman Farm of Washington State University (WSU) at Pullman, Washington in 1996. This nursery consisted of 61 lines planted in single-row plots 6 m in length, with a spacing of 30 cm between the rows. Twenty grains were sown in each row, but due to unfavorable conditions in the field only 738 plants were harvested. Eighteen homozygous lines (pedigree: Table 1) were selected and propagated in the field at Pullman in 1997 with the same parameters as in 1996.

Harvested T_4 and T_5 material was replanted in irrigated drill strips of 9.3 or 46.5 m^2 at the WSU experimental station at Royal Slope, Washington in 1998 with a seeding rate of 11 $g \cdot m^{-2}$ (15 cm

Table 1 Pedigree of transgenic plants

Plant generation						Recombinant enzyme ($\mu g \cdot mg^{-1} \cdot$protein)
T_0	T_1	T_2	T_3	T_4	T_5	
	1995/96	1996	1997	1998	1999	
6.2.2	6.2.2.G7	G7a 1–96	19001–076	5601		0.11–0.22
6.2.2	6.2.2.G8	G8a 1–96	19077–264	5602		0.07–0.16
6.2.2	6.2.2.G9	G9a 1–96	19265–341	5603		0.24–0.32
6.2.1	6.2.1.5	13133–138	19547–567	5605		0.05–0.09
6.2.2	6.2.2.2.	13115				
6.2.2	6.2.2.21	13139–144	19568–601	5615		0.23–0.33
6.2.2	6.2.2.40	13146–151	19602–692	5606		0.31–0.40
6.2.2	6.2.2.25	13157–161	19722–733	5617		0.15–0.19
8.2.1	8.2.1.23	13101–106	19769–860	5607/5608	Othello and Burley	0.52–1.21
8.2.1	8.2.1.25	13107–111	19861–888	5609	Pullman and Burley	0.49–1.12
8.2.1	8.2.1.19	13112–114	19889–921	5610	Othello	0.57–0.78
	1995/96	1996	1996	1997	1998	
6.2.2	6.2.2 GH7a	GH7a 1–10	13163–172	19351–413	5611	0.05–0.20
6.2.2	6.2.2 GH8a	GH8a 1–10	13173–182	19430–452	5612	0.04–0.15
6.2.2	6.2.2 GH9a	GH9a 1–16	13184–189	19453–467	5613	0.12–0.22
6.2.2	6.2.2 GH7b	GH7b 1–10	13190	19734–749	5618	
6.2.2	6.2.2 GH9b	GH9b 1–16	13191–199	19750–768	5619	0.15–0.19
6.2.2	6.2.2.4	6.2.2.4.9	13116–120	19471–517	5604	0.25–0.27
6.2.2	6.2.2.3	6.2.2.3.8	13121–125	19521–547	5614	0.11–0.12
6.2.2	6.2.2.3	6.2.2.3.5	13126–133	19922–925	5620	0.11–0.36
6.2.2	6.2.2.3	6.2.2.3.4	13152–156	19693–721	5616	0.05–0.15

space between rows). In 1999 T_5 transgenic lines expressing high amounts of recombinant enzyme were grown in large-scale propagations at three different locations: Pullman, Washington (929 m^2); Othello, Washington (1171 m^2) and Burley, Idaho (1393 m^2). Experimental fields were surrounded by a belt of untransformed barley plants in all years except 1998, when oat plants were used. Rows were harvested by hand, whereas drills were harvested using a small-plot combine.

Crosses

Plants for propagation and crossing of the transgenic plants with elite cultivars were grown in greenhouse maintained at 21°C (day) and 16°C (night), 16-h light (photon flux density of 200 $\mu E \cdot m^{-2} \cdot 5^{-1}$) and 8-h dark, respectively. Plants were watered every second day with water supplemented with 100 ppm 20N:20P:20K greenhouse fertilizer.

Southern and Western blot analysis

Genomic DNA from leaf tissue of 4-week-old plants was isolated and purified with the DNeasy Plant Mini Kit (QIAGEN); 10-µg aliquots were then digested with *Eco*RI and *Pst*I, separated on a 0.8% agarose gel, blotted onto a positively charged nylon membrane and probed with the [^{32}P]-labeled coding region of the heat-stable (1,3–1,4)-β-glucanase gene (620 bp). The procedure used for Western blot analysis has been described by Horvath et al. (2000).

Activity assays and segregation analysis

Segregations were assessed by scoring transgenic generations with multiplex polymerase chain reaction (PCR) analysis and enzyme assays as described in Horvath et al. (2000). The eight oligo nucleotide primers used for PCR amplification are as follows:

D13GCU (5′-GTTCGACTGCGCCGAGTACCGCAGC-3′);
D13GCL (5′-GGCGTAGGTGTGGAAGCCCTTGCTG-3′);
BARU (5′-CATGCCGGCGGTCTGCACCATCGTC-3′),
BARL (5’-CCTCGTGCATGCGCACGCTCGGGTC-3’);
GUSU (5′-CGTCCTGTAGAAACCCCAACCCGTG-3′);
GUSL (5′-TCACCATTCCCGGCGGGATAGTCTG-3′);
EIIU (5′-ATCGGGGTGTGCTACGGCATGAGCG-3′);
EIIL (5′-TGTTGGCCATGAGCGGCGCGTTGGT-3′).

Primers D13GCU and D13GCL amplify a 271-bp fragment of the open reading frame for the heat-stable (1,3–1,4)-β-glucanase (Jensen et al. 1996), BARU and BARL amplify a 365-bp internal fragment of the *bar* gene (White et al. 1990), GUSU and GUSL amplify a 439-bp fragment of the *gus* gene (Jefferson et al. 1987) and EIIU and EIIL amplify a 509-bp fragment from the coding region of the endogenous barley (1,3–1,4)-β-glucanase isoenzyme II gene (Wolf 1991).

Heterozygous and homozygous plants were identified with the zymogram assay for recombinant heat-stable β-glucanase on individual germinated kernels, and the amount of recombinant enzyme was measured by the azo-barley glucan assay (Megazyme). Recombinant and endogenous enzyme in mature grain was analyzed separately in the endosperm and embryo. β-Glucuronidase activity was determined by histochemical staining, and the expression of the *bar* gene was monitored by the colorimetric seedling test for phosphinotricin transacetylase activity. These methods are described in detail in Horvath et al. (2000).

Micro-malting

Samples (37 g) of barley cvs. Golden Promise and Harrington and the selected 18 homozygous transgenic lines were malted and kiln-dried in a micromalting unit developed at WSU as follows: 48 h of steeping at 13°/14°C (8 h aerated water, 16 h humid air, 24 h aerated water), 96 h of germination at 11°–13°C and 12 h of kiln drying (6 h 50°–55°C, 2 h 50°–80°C, 4 h at 80°C). Enzyme activities were determined for the recombinant as well as for the endogenous barley β-glucanase and for α-amylase as described previously (Horvath et al. 2000).

Results

Stability of transgene expression and field performance

The amount of heat-stable enzyme produced in grains of the 18 homozygous transgenic lines germinated for 5 days at 16°C was determined for grain produced in 1996, 1997 and 1998 (Fig. 1). On the basis of their enzyme levels, transgenic lines were classified into high-, intermediate- and low-expressing lines. The expression level for each line was consistent over all 3 years. The three high-expressing lines 5607/8, 5609 and 5610 (Fig. 1, Table 1) were derived from the T_0 primary transformant 8.2.1 and produced 0.49–1.21 µg heat-stable β-glucanase per milligram soluble protein. An increase in enzyme formation was observed over the 3-year period, which was due to an improvement in field conditions. This trend was also reflected in increased 1000-grain weights (Fig. 2A). Lines obtained from T_0 transgenic plants 6.2.1 and 6.2.2 showed a limited variation in recombinant enzyme production: low expression ranged

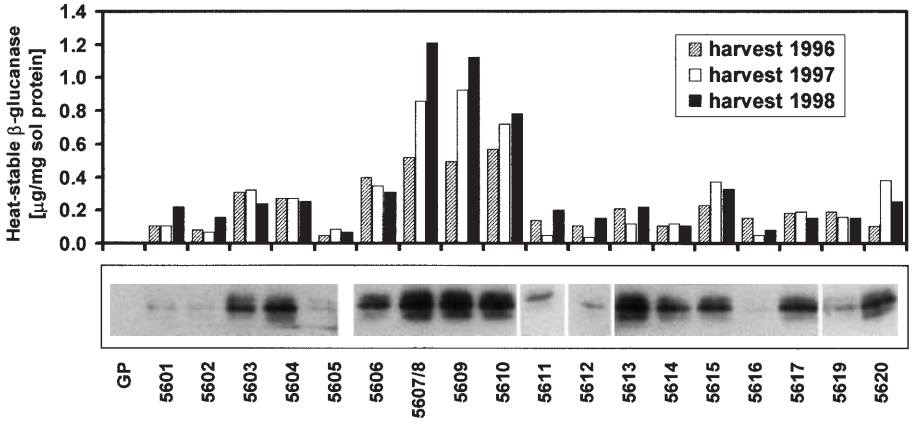

Fig. 1 Expression of recombinant heat-stable (1,3–1,4)-β-glucanase in germinated grains of homozygous transgenic lines in 1996, 1997 and 1998. The amount of enzyme protein recognized by monoclonal antibody in the Western blots from 1998 (*below the bar graph*) correlates approximately with enzyme activity. The three protein isoforms (M_r=28, 30 and 32 kDa) differ in *N*-glycosylation. *GP* Golden Promise, *5601–5620* transgenic lines

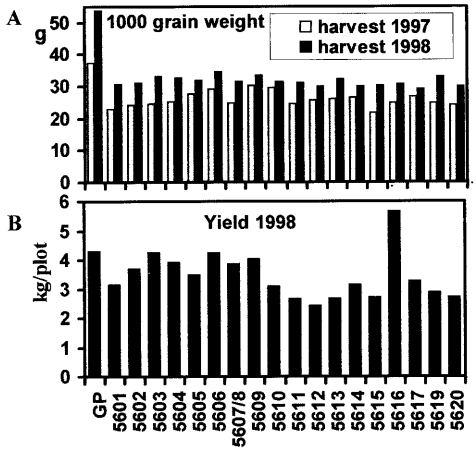

Fig. 2 **A** One thousand-grain weights in wild-type Golden Promise and in transgenic lines compared for 1997 and 1998. **B** Yield in 1998 per 8.4-m^2 plots

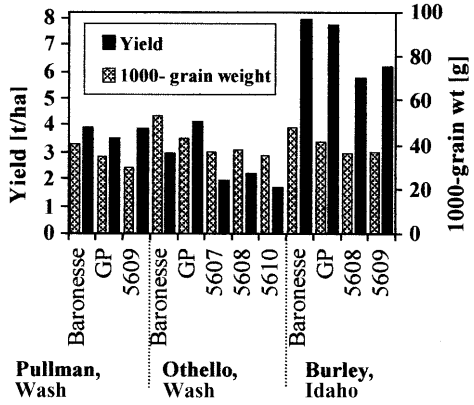

Fig. 3 One-thousand grain weights and yields per hectare are compared at three different locations for Baronesse, Golden Promise (*GP*) and transgenic lines expressing high levels of the heat-stable (1,3–1,4)-β-glucanase during germination

from 0.04 to 0.22 µg·mg^{-1} protein (lines 5601, 5602, 5605, 5611, 5612, 5616, and 5619) and intermediate levels from 0.11 to 0.40 µg·mg^{-1} protein (lines 5603, 5604, 5606, 5613, 5614, 5615, 5617, 5619 and 5620). Even the lowest production of 0.04 µg heat-stable β-glucanase·mg^{-1} protein was significant since untransformed Golden Promise did not show any activity at all.

Western blot analysis on germinated grains of transgenic lines from field trials in 1998 revealed that the production of active enzyme correlates with the amount of recombinant protein (Fig. 1). Three major bands of 28, 30, and 32 kDa were recognized by the heat-stable β-glucanase-specific monoclonal antibody, whereas only one 24-kDa band was observed for the unglycosylated recombinant protein produced in *E. coli*. The retarded mobility forms have also been observed for the enzyme produced in aleurone protoplasts (Jensen et al. 1996) and have been analyzed with the enzyme expressed in yeast. They are due to alternate signal peptide cleavage sites and different *N*-glycosylations. Of the three possible glycosylation sites only Asn 31 and Asn 185 are glycosylated, while Asn 40 is not. Site-directed mutagenesis of Asn to Gln suggested that steric hindrance at sequon Asn 40 by the N-glycans of the other two sequons causes the absence of glycosylation at this amino acid (Meldgaard and Svendson 1994; Meldgaard et al. 1995).

Agronomic characteristics of transgenic lines and Golden Promise were compared in 1997 and 1998 (Fig. 2). The average 1000-grain weight of transgenic lines was 25.8±0.5 g in 1997 and 31.6±0.4 g in 1998. This is a 31% and 41% weight reduction compared to Golden Promise in 1997 and 1998, respectively. Nevertheless, the yield of transgenic lines in 1998 was on average comparable to Golden Promise (4.32 kg per 8.4-m^2 plot). There was no correlation between yield and amount of enzyme produced.

Transgenic lines producing high levels of recombinant β-glucanase under the control of the α-amylase promoter as well as Golden Promise (GP) and Baronesse were propagated on a larger scale in 1999 in three different locations to investigate the effect of environmental conditions on 1000-grain weight and yield (Fig. 3). In the dry climate of Pullman, the 1000-grain weight of Baronesse was 40.6 g compared to 29.7 g of the transgenic Golden Promise line 5609. Nevertheless, lines 5609 and Baronesse yielded 3.8 and 3.9 t·ha^{-1}, respectively. Under irrigated conditions in Othello, Washington, wild type Golden Promise performed unexpectedly superior to Baronesse, untransformed Golden Promise yielded about double the amount of that of the transgenic lines, 4.1 t·ha^{-1} compared to 2.0, 2.2 and 1.7 t·ha^{-1}. Transgenic lines 5607/8 and 5609 showed reduced kernel plumpness (data not shown) as reflected in the 1000-grain weight. The best field performance for all lines was observed in the irrigated field at Burley, Idaho. Baronesse and Golden Promise gave comparable yields of 7.9 and 7.7 t·ha^{-1}, respectively, whereas transgenic lines 5608 and 5609 gave 5.8 and 6.2 t·ha^{-1}, respectively. This shows the dramatic impact of different conditions on the field performance of wild-type cultivars and the transgenic barley lines.

Micro-malting

In micro-malting experiments with the 18 homozygous lines, the synthesis of enzymes during malting and survival after kiln drying with temperatures of up to 80°C were determined. Representative results of micro-malting with Golden Promise and 4 transgenic lines are presented in Fig. 4. One line is characteristic of high (5609), two of intermediate (5604, 5606) and one of low expression (5616). An increase in heat-stable β-glucanase was observed in the high-expressing line, reaching its maximum of 1.4 µg·mg^{-1} protein after 96 h of germination (Fig. 4A). For intermediate-expressing lines an in-

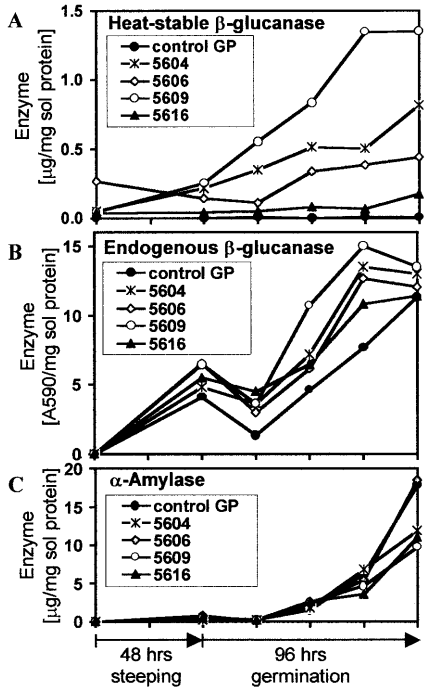

Fig. 4A–C Micro-malting of Golden Promise and 4 representative lines transgenic for the heat-stable (1,3–1,4)-β-glucanase (*5616*=low-, 5604 and *5606*=intermediate- and *5609*=high-expressing lines): time course of recombinant heat-stable (1,3–1,4)-β-glucanase (**A**), endogenous barley β-glucanase (**B**), and barley α-amylase (**C**)

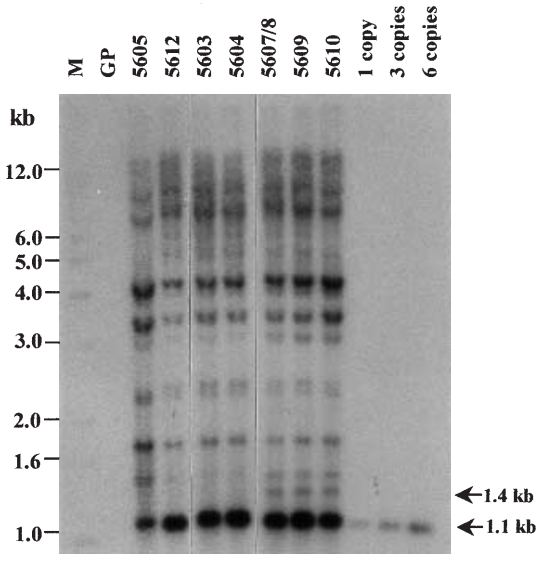

Fig. 5 Southern blot hybridization of 7 homozygous lines expressing the heat-stable (1,3–1,4)-β-glucanase gene. The DNA was digested with *Eco*RI and *Pst*I, separated on an 0.8% agarose gel, blotted on a positively charged nylon membrane and probed with the [^{32}P]-labeled coding region of the heat-stable (1,3–1,4)-β-glucanase gene (620 bp). *GP* Golden Promise, *5605* low-expressing line derived from primary transformant 6.2.1, *5620, 5603, 5604* intermediate-expressing lines originating from T_0 plant 6.2.2, *5607/8, 5609, 5610* high-expressing lines obtained from primary transformant 8.2.1, *Lanes* at *right*: one, three and six copies of digested plasmid DNA

crease of up to 0.7 µg·mg^{-1} protein was determined, while no enzyme induction was observed in low-expressing lines. Unexpectedly, low amounts of active heat-stable enzyme, ranging from 0.05 to 0.27 µg·mg^{-1} protein, were present in the ungerminated mature grain in the barley embryo but not in the endosperm. This indicates that the α-amylase promoter is leaky in the transgenic plants, possibly because of an unsuitable locus of insertion of the transgene in the chromosome or the high copy number. The production of endogenous barley (1,3–1,4)-β-glucanase during germination followed the same time course in Golden Promise and the transgenic lines (Fig. 4B), whereas α-amylase activity during germination was slightly reduced in some of the transgenic lines (Fig. 4C). After kiln drying for 6 h at 45°C and 4 h at 80°C 16% of the endogenous β-glucanase of Golden Promise survived and 40% of that of cv Harrington. In the transgenic lines a survival level of 11–29% was determined for the endogenous β-glucanase, while the heat-stable (1,3–1,4)-β-glucanase survived at levels of 70–100%. Between 70% and 80% of the α-amylase survived in Golden Promise and Harrington, and 40–70% survived in the transgenic lines.

Southern Blot hybridization

Southern blot analysis of digested genomic DNA of the transgenic lines revealed complex restriction fragment patterns (Fig. 5). As expected, progenies originating from the same T_0 transformant showed the same patterns of fragment sizes. However, it was surprising that the lines derived from the 6.2.1, 6.2.2, and 8.2.1 independent transformation events showed similar fragment patterns. The smallest band (1.1 kb) was identified as the intact transgene and was observed in all transgenic lines. The 1.1-kb fragment consisted of 92 bp of the 3′ end of the α-amylase promoter, the code for the α-amylase signal peptide, the complete coding region for the heat-stable β-glucanase and the *nos* terminator. Based on the known copy number of digested plasmid DNA, an estimated number of 15–20 copies of the intact transgene were integrated into the genome of all transgenic lines except line 5605 (originated from T_0 plant 6.2.1), which contains about 10 copies. This line has a very low expression level. In addition, multiple fragments ranging from 1.4 kb to 8.8 kb were observed, which reflects the complex integration pattern of the transgene. Although the patterns of the low, intermediate- and high-expressing lines were similar, there was a distinct 1.4 kb band that was only present in the high-expressing lines originating from primary transformant 8.2.1. The Mendelian segregation of the transgenic plants suggests that the transgene copies were integrated in a single genetic locus as has been documented for other cereal transformants; for example in transgenic oats (Pawlowski and Somers 1998) and rice (Kohli et al. 1998).

Recombination breeding with the transgene

The yield limitations, the diminished plumpness of the transgenic grain, and the poor adaptation of Golden Promise to the drylands of the Pacific Northwest (USA) led us to investigate if these characteristics could be improved by transferring the transgene into other genotypes. Crosses were made with the three types of transgenic lines (cf. Fig. 1): (1) lines expressing 0.5–1.2 µg enzyme per milligram soluble protein (5607, 5609); analyses have been so far carried out to the F_3 generation; (2) line 5603 expressing 0.3 µg enzyme and (3) line 5601 expressing 0.1–0.2 µg enzyme. The latter 2 crosses have been analyzed up to the F_5 generation.

The recombinant high-enzyme-producing line 5609 was crossed with the malting barley cultivars Galena and B1202. Lines 5609 and 5607 were crossed with the proanthocyanidin-free lines Ca803111 [*ant* –499(Apex)× Alexis] and Ca803803 [Secobra 16738×*ant* –499(Apex)]. The F_2 generations were grown in the greenhouse, and from each F_2 plant 20 grains were analyzed with the zymogram assay, which identified the homozygous, heterozygous and the wild-type individuals with respect to transgene expression (Table 2). In 3 of the F_2 populations agreement with a 1:2:1 segregation was observed, whereas in 2 populations a significant excess of homozygous transgenic individuals was recorded. Such segregation anomalies are due to environmentally conditioned, uneven gametic selection and are a common occurrence in barley crosses (Henningsen et al. 1993). Heterozygous F_2 plants segregated in 3 populations with the expected 3:1 ratios, while a deficit of untransformed progeny was present in the other 2 populations (Table 2).

In the field-grown F_2 generation from the above crosses, a distinct segregation of four morphological phenotypes was recognizable, as illustrated in Fig. 6B–F. The four types were: Golden Promise, with its dwarf, short-awned phenotype; erect plants with dense spikes of intermediate height; high erect plants with dense basal rachis internodes; nutans types with long straw and drooping spikes (Fig. 6B, 2nd to 5th plant from right). The original transgenic plants were slightly shorter than Golden Promise (Fig. 6B right-most plant) but could not always be distinguished from Golden Promise in the progeny. Therefore, the two categories were lumped into one class. These four morphological classes were observed and their frequencies determined in all crosses with the transgenic plants. We designated them nutans (nut), high erect (hert), erect (ert) and ari-e. The latter designates Golden Promise phenotypes. Segregations in the F_2 for 8 crosses with transgenic lines 5607, 5609 and 5610 are presented in Table 3.

An understanding of these clear-cut morphological segregations derives from the origin of cv. Golden Promise, which is a γ-ray-induced mutant of the nutans cv. Maythorpe (Sigurbjørnsson and Micke 1969). It results from a recessive mutation in the breviaristatum gene *Ari-e* on the short arm of chromosome 7 (5H) (Thomas et al. 1984; Franckowiak 1991). The mutant is allelic to the independently induced mutants *ari-e*^{1}, *ari-e*^{119}, *ari-e*^{156} and *ari-e*^{228} (Kucera et al. 1975). DNA of Golden Promise and Maythorpe was analyzed with some 300 randomly amplified polymorphic DNAs (RAPDs) and no difference detected, while an amplified fragment length polymorphism analysis (AFLP) detected a band shift, suggesting a single base difference between the two cultivars (Pakniyat et al. 1996). The Golden Promise a*ri-e* gene has been bred into spring barley cultivars like Midas, Goldmarker, Chad and Tyne (Pakniyat et al. 1997), but not into the cultivars and lines used in the present crosses.

Table 2 F_2 and F_3 segregation of the transgene expressing the heat-stable (1,3–1,4)-β-glucanase with the α-amylase promoter in the germinating grain (*WT* wild type. *Hom*. homozygous. *Het*. heterozygous)

Cross	F_2 plants					F_3-segregation			
	Transgenic		*WT*	χ^2 (1:2:1)	*P*	*Transgenic*	*WT*	χ^2 (3:1)	*P*
	Hom.	Het.	Hom.			Hom./het.	Hom.		
5607×Ca803111	21	27	20	2.9	0.2	396	132	0	1
5609×B1202	18	35	21	0.5	0.8	542	166	0.9	0.7
5609×Ca803111	36	26	11	23.2	<0.001	389	123	0.3	0.5
5609×Ca803803	23	33	19	1.5	0.5	476	121	7.1	<0.01
5609×Galena	32	34	10	13.6	<0.001	541	143	6.1	<0.01

Table 3 Morphological segregation in the F_2 generation from crosses of transgenic lines expressing the heat-stable (1,3–1,4)-β-glucanase with modern cultivars (Fig. 6B) [*nut* nutans (see Fig. 6E). *hert* high erect (see Fig. 6F). *ert* erect (see Fig. 6D). *ari-e* Golden Promise (see Fig. 6C)]

Cross	nut	hert	ert	ari-e		χ^2	*P*
5610×Galena	40	82	82	61	For 3:5:5:3	4.48	~0.20
5607×B1202	40	71	79	36		2.09	~0.60
5607×Ca803111	40	100	88	46		5.54	~0.15
5610×*ant*29–2110	38	104	69	54		10.84	~0.025
5609×Ca803803	32	87	58	19	For 3:7:5:1	4.52	~0.20
5609×B1202	48	106	68	23		4.92	~0.15
5609×Ca803111	32	104	101	23		14.20	~0.001
5609×Galena	55	127	91	18		0.006	~1

Fig. 6 **A** Propagation of T_5 generation and Baronesse in field trial 1999 at Othello, Washington, **B** Crosses of transgenic Golden Promise plants with elite cultivars segregated into four morphological phenotypes (from *left* to *right*): nutans, high erect, erect, *ari-e*, and original transformant. **C** Short-awned (*breviaristatum*, *ari-e*) phenotype of Golden Promise, **D** dense spike of erect plant, **E** drooping spike of nutans phenotype, **F** high erect

The segregation ratios observed appear to fit modified dihybrid models. In 4 of them there is agreement with a segregation of 3 nutans: 5 high erect: 5 erect: 3 ari-e (Golden Promise) and in the other 4 with a segregation of 3:7:5:1, respectively (Table 3). This suggests that the cultivars used in the present crosses contain another major semidwarf gene responsible for the erect phenotypes.

Since lines homozygous for the four different phenotypes were obtained in the crosses discussed below, one might consider the following model for putative genotypes of the two different dihybrid segregations:

3 nutans:	1*nut nut Ari Ari* 2 *Nut nut Ari Ari*
7 high erect:	1*Nut Nut Ari Ari* 4 *Nut nut Ari ari* 2 *Nut Nut Ari ari*
5 erect:	1*Nut Nut ari ari* 2 *nut nut Ari ari* 2 *Nut nut ari ari*
1 ari-e	1 *nut nut ari ari*

Table 4 F_2 and F_3 segregations of the transgene expressing the heat-stable (1,3–1,4)-β-glucanase with the α-amylase promoter in the germinating grain of plants with the four different morphological phenotypes (*WT* wild type. *Hom.* homozygous. *Het.* heterozygous)

Phenotype	F_2 plants					F_3 segregation			
	Transgenic		*WT*	χ^2 (1:2:1)	*P*	*Transgenic*	*WT*	χ^2 (3:1)	*P*
	Hom.	Het.				Hom./het.	Hom.		
5607 ×B1202									
nutans	8	14	7	0.10	~0.95	230	50	7.6	~0.005
high erect	11	15	4	3.2	~0.05	231	69	0.64	~0.70
erect	9	13	4	1.9	~0.15	219	41	12.1	~0.0005
ari-e	3	4	2	0.48	~0.68	68	12	16.3	~0.0005
5610×Galena									
nutans	6	9	14	8.6	~0.005	148	32	5.01	~0.025
high erect	2	5	14	18.1	<0.001	81	39	3.6	~0.06
erect	6	12	10	1.7	~0.21	193	67	0.08	~0.75
ari-e	2	3	1	0.33	~0.76	40	20	2.23	~0.15

Table 5 Analysis of crosses of a line expressing 0.3 µg and of a line expressing 0.1–0.2 µg recombinant enzyme with different cultivars. Leaf samples of F_2 plants were analyzed with primers for the heat-stable (1,3–1,4)-β-glucanase gene by PCR. The values (in micrograms) designate the amount of recombinant enzyme per milligram soluble protein expressed in the malt of the line

Cross		PCR +		PCR –		χ^2 3:1	*P*
		Observed	Expected	Observed	Expected		
Ca 2110[a]×5603	(0.31 µg)	466	522	230	174	12.01	~0.0005
Baronesse×5601	(0.13 µg)	43	25	51	17	6.01	~0.025
5601×Baronesse	(0.16 µg)	69	73.5	29	24.5	1.10	~0.30
Caminant×5601	(0.13 µg)	105	116.25	50	38.75	4.35	~0.04
Baronesse×5601	(0.12 µg)	51	63.75	34	21.25	10.2	~0.001
Baronesse×5601	(0.16 µg)	192	198.75	73	66.25	0.91	~0.30

[a] Ca 2110 is proanthocyanidin-free mutant *ant* 29-2110

and

3 nutans: 1*nut nut Ari Ari* 2 *Nut nut Ari Ari*
5 high erect: 1*Nut Nut Ari Ari* 4 *Nut nut Ari ari*
5 erect: 1*Nut Nut ari ari* 2 *nut nut Ari ari* 2 *Nut Nut Ari ari*
3 ari-e: 1*nut nut ari ari* 2 *Nut nut ari ari*

Considering the morphological analyses of breviaristatum and erectoides mutations (Kucera 1975; von Wettstein 1954; Gustafsson and von Wettstein 1958; Persson and Hagberg 1969) the types of interactions assumed in the two models are feasible. Both lengthening and shortening of rachis internodes as well as lengthening and shortening of culm internodes were observed with these genes, and heterozygotes can show dominance in culm internodes and recessivity in rachis length and vice versa. The observation of two dihybrid ratios might be accounted for by the introduction of modifying genes from the modern cultivar parent and also by the large quantitative variation, especially in straw length and internode distributions, caused by environmental fluctuations. Of particular was that the height of the F_1 plants within a given cross could vary from 39 to 98 cm, although a statistical analysis for segregation among different height groups did not reveal any significant differences.

Plants of the four morphological phenotypes in the F_2 generation were scored using the zymogram assay (Table 4). The transgene segregated approximately in a 1:2:1 ratio in most phenotypes. There is thus no indication that the insertion of the transgene is linked to the *ari-e* locus or the semidwarf gene of the cultivars.

Line 5601 expressing 0.1–0.2 µg (1,3–1,4)-β-glucanase per milligram soluble protein during germination was crossed with Baronesse and Caminant, and line 5603 expressing 0.3 µg recombinant enzyme with Ca 2110. Leaf samples of plants from 6 F_2 progenies were analyzed by PCR with primers for the heat-stable (1,3–1,4)-β-glucanase gene to evaluate the segregation of the transgenes. The F_2 plants segregated either normally or with a deficit of PCR-positive plants (Table 5).

Grain of the PCR-positive homozygous and heterozygous F_2 plants were planted in an F_3 field nursery in 1999, and the individual plant progenies were analyzed for morphological segregations. From the dihybrid model we expected that the four homozygous genotypes would occur in the F_2 at a frequency of 1 in 16. Thus, in the cross Ca 2110×5603, 28.4 F_2 plants should be homozygous for each of the morphological types, and for the other 2 crosses (Baronesse×5601 and Caminant×5601) 22 and 6.9 plants, respectively. The frequencies observed are in reasonable agreement with this expectation (Table 6).

From the homozygous F_3 rows characteristic plants were harvested and their grains analyzed using the zymogram test for the heat-stable (1,3–1,4)-β-glucanase. One nutans plant was homozygous for the transgene (36:0) and 3 were negative for the transgene (0:102). Among 10 high erect plants 3 were homozygous for the transgene (85:0), 5 heterozygous (115:42) and 3 lacked

Table 6 Frequencies of morphological phenotypes in the F_2 of three crosses of transgenic lines with three cultivars

Segregations of F_2 plants in F_3	Ca 2110[a]×5603	Baronesse×5601	Caminant×5601
Homozygous nut	26 (267)	17 (208)	12 (130)
Homozygous hert	32 (304)	37 (443)	5 (58)
Homozygous ert	18 (178)	16 (183)	7 (80)
Homozygous ari-e	33 (343)	14 (160)	4 (33)
hert, ert	69	102	19
hert, ert, ari-e	85	54	19
ert, ari-e	72	33	13
nut, hert, ert	30	16	6
nut, ert	24	17	7
hert, ari-e	17	11	0
nut, ert, ari-e	15	8	5
nut, hert	14	13	5
nut, hert, ert, ari-e	10	4	2
nut, hert, ari-e	6	7	2
nut, ari-e	4	3	4
Sum	455	352	110

[a] Ca 2110=*ant* 29-2110
[b] Number in brackets: F_3 plants counted to assess the genotype of the F_2 plant

the transgene (0:99). One erect plant was found to be homozygous for the transgene (36:0). Among the homozygous ari-e plants a heterozygote for the transgene (28:9) and a homozygous negative plant (0:33) were identified. It was concluded that the transgene can be expressed in the plants with the nutans, the high erect and erect growth habits.

In order to determine if relocation of the transgene into a different genotype can change the 1000-grain weight, we analyzed F_4 lines of the cross Caminant×5602 with the nutans and high erect phenotype. With respect to transgene expression, homozygous, heterozygous or wild-type plants were identified by the zymogram test of 20–25 kernels. Ten nutans plants homozygous for the transgene had an average 1000-grain weight of 41.0± 0.93 g, while 12 nutans lines lacking the transgene yielded a 1000-grain weight of 40.4±1.12 g. Thus, a grain weight improvement in the transgenic grains was observed in this case. A comparison of the 1000-grain weights of 6 high erect plants with the transgene and 5 plants without the transgene gave weights of 41.3±0.849 and 50.4±2.66 g, respectively. While a clear increase compared to the original transgenic plants was observed, the plants without the transgene were superior.

Discussion

Expression levels of the protein-engineered (1,3–1,4)-β-glucanase in $T_{2/3}$-T_5 generations of transgenic barley plants remained stable over a 3-year period of field trials and in the three lines expressing high enzyme levels stability was proven over 4 years. Among cereal crops, transgene expression and inheritance over three to five generations has been studied for barley (Jensen et al. 1998), maize (Armstrong et al. 1995), wheat (Demeke et al. 1999), and rice (Chareonpornwattana et al. 1999). In the latter, transcriptional silencing of the transgene occurred sooner or later in all transformants.

Transformation of the barley cultivar Golden Promise has become a standard procedure (Wan and Lemaux 1994; Tingay et al. 1997), but transforming different barley varieties still requires the bombardment or co-cultivation with *Agrobacterium* of a very large number of immature embryos. The poor field performance of Golden Promise in the dryland areas of the Pacific Northwest make single-seed descent, double-haploid production or other cross breeding procedures a requirement in order to introgress the transgene into a genetic background lacking the *ari-e* semidwarf mutation. Demeke et al. (1999) reciprocally backcrossed transgenic wheat T_4 and T_5 lines expressing the GUS reporter gene with their host cultivar Fielder (Demeke et al. 1999). The reporter gene *uidA* was detected in all F_1 plants and the hybridization patterns of the Southern blots of the F_2 plants were indistinguishable from the original T_1, and thereof derived T_5 plants, indicating that no rearrangement had occurred in the progeny of successive generations. This documented that transgenes introduced by the biolistic method insert in a single locus of a plant chromosome, as has been previously shown in other studies (e.g. Wan and Lemaux 1994).

Field studies of non-transformed barley lines regenerated by tissue culture have frequently displayed considerable numbers of mutations (somaclonal variation). Some cultivars displayed high frequencies of somaclonal variation, reducing plant height, yield and kernel plumpness (Ullrich et al. 1991; Bregitzer and Poulson 1995), while other cultivars had low frequencies of somaclonal variation (Baillie et al. 1992). Reduced height and lowered 1000-grain weight are also characteristic of transformants in Golden Promise (Bregitzer et al.1998) but could be improved in our experiments by removing the *ari-e* semidwarf mutation upon cross breeding with cultivars of a very different genotypic constitution. It remains to be analyzed if such an improvement is due to the selection of recombinant plants combining the transgenic locus with modifying genes or by segregating away negative mutant genes induced by tissue culture.

We have derived, from a given original transformant, stable transgenic lines that differ in recombinant enzyme expression (e.g. 5603 and 5602, cf. Table 1). These are

considered to be due to early rearrangements of the transgenic locus in cells forming the somatic embryo. In Southern hybridizations of the DNA of T_5 plants, bands of larger restriction fragments were present in addition to the expected 1.1-kb fragment of the heat-stable β-glucanase gene. The presence of these fragments suggests rearrangement of the introduced DNA leading to interspersion of the transgene with plant and/or plasmid DNA, as has been observed in transgenic rice (Kohli et al. 1998) and oats (Pawlowski and Somers 1998). Interestingly, all transgenic barley plants derived from three independent primary transformants showed very similar patterns of the larger restriction fragments, but produced significantly different amounts of recombinant enzyme. Isolation and sequencing of the transgenic locus is required to determine if the differences resulted from alterations in the structure of the multiple copies of the β-glucanase gene or from the scrambling of the introduced genes with the DNA of the host chromosome.

All three transgenes were detected by multiplex PCR reactions amplifying partial fragments of the coding regions. Plants contained either all three or none of the transgenes. A 438-bp fragment of the coding region of the *uidA* gene was also present in the high ß-glucanase expressing lines, but histochemical staining for GUS in germinating seedlings or pollen was negative as early as in the T_2 generation. The *uidA* gene is under the control of the synthetic pEmu promoter, which directs high levels of constitutive gene expression in monocots (Last et al. 1991). It is remarkable that plants with so similar patterns in Southern blot analysis are not only different in the expression levels of the analyzed intact β-glucanase gene but also for GUS expression. GUS staining was readily detected in all lower β-glucanase-expressing transgenic lines. Differences in expression of the reporter gene, the selectable marker gene and target gene have been frequently described.(e.g.Yao et al. 1997; Chareonpornwattana et al. 1999). Further investigations will have to show whether this is a case of gene truncation or transcriptional or translational silencing.

In all transformants, the promoter of the high-pI α-amylase gene became leaky, thereby expressing the heat-stable (1,3–1,4)-ß-glucanase ectopically in the embryo of the developing grain. In many cases it was no longer inducible during germination. It is likely that this is due to an unsuitable insertion into chromatin that is transcriptionally inactive in aleurone cells of germinating grains.

The micro-malting experiments demonstrated that transgenic barley lines can develop, during malting, a protein-engineered (1,3–1,4)-β-glucanase with the same profile as the endogenous hydrolytic enzymes secreted from the aleurone into the endosperm. Such transgenic lines can thus be applied to use as kilned malt in processes that make action of the enzyme in mashing procedures desirable. We also found that such optimal lines have to be selected among several to many initial transformants.

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