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## Transformation breeding of Barley: towards malting quality improvement

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# Abstract

Transformation using *Agrobacterium tumefaciens* was used to generate transgenic barley lines which were developed and tested over several generations. After a preliminary study of transformed lines, a methodology to produce selectable marker-free (SMF) transformed plants was adapted and implemented to insert transgenes aimed at improving barley malting quality.

The strategy was to increase enzyme gene copy number by transforming barley with transgenes encoding three malting enzymes - low- and high-isoelectric point (pI)  $\alpha$ -amylase, and  $\alpha$ -glucosidase. All three transgenes were inserted in an expression cassette driven by a barley high-pI  $\alpha$ -amylase promoter, which was itself inserted into one T-DNA of a "twin T-DNA" transformation plasmid. After individual insertion of transgenes and removal of the selectable marker gene by segregation, transgenes were introgressed from the transformation recipient, Golden Promise, into the Australian cultivar Sloop by one to four backcrossing generations.

To test for enzyme augmentation, sibling plants null or homozygous for transgene presence were grown and their aleurones tested for enzyme production after exposure to gibberellic acid in the laboratory. While one experiment with an early transformed line showed an increase in  $\alpha$ -amylase isoform production in response to several transgene copies, five introgressed transgenic lines with one or two transgene copies showed no detectable increase. In contrast, a line transformed with one  $\alpha$ -glucosidase transgene copy showed a significant increase in  $\alpha$ -glucosidase production. This difference is probably a consequence of endogenous gene copy number,  $\alpha$ -amylase being encoded by about nine copies, and  $\alpha$ -glucosidase by only one.

# Introduction

Although the future of transgenic barley is presently uncertain in the light of public controversy surrounding genetically modified organisms, its potential for adding to the barley gene pool is such that research on possible applications remains desirable.

Application of barley transformation technology in producing potentially useful transgenic crops is now showing considerable promise. Papers have reported encouraging results in expressing bacterial (von Wettstein et al., 2000) or fungal (Nuutila et al., 1999; Patel et al., 2000) genes in barley to enhance its use for malting and/or feed purposes, and also raise the potential for the use of barley as a bioreactor to grow (and then purify) large quantities of useful protein (Schunmann et al., 2001).

Stable transformation of barley was first reported in 1994 (Wan & Lemaux, 1994), and since then there have been many reports of successful barley transformation (for review see Lemaux et al., 1999). Most studies have used microparticle bombardment for transgene insertion, but the use of *Agrobacterium tumefaciens* for transformation of barley (Tingay et al., 1997) offers an alternative methodology. While employing tissue culture of immature scutella in the same way as the first biolistic technique, in our hands the *Agrobacterium* technique is considerably more efficient. It also appears to offer lower occurrence of multiple transgene insertion, a problem which has been associated with gene silencing, and also infrequent (if any) polyploidy in transformants, a problem in biolistic transformation (Choi et al., 2000).

An important objective of this project has been to explore strategies by which this transformation technology can be adopted, with associated procedures for assessing transgene expression, stability and heritability, to create improved barley germplasm in a form amenable to progressing the pathway of delivery to industry. To aid consumer acceptance of such transgenic cultivars an ancillary objective was to generate transgenic lines which were free of antibiotic resistance genes, thereby making these barleys more environmentally responsible and consumer friendly. These strategies have been explored using transgenes coding for diastatic enzymes.

Production of the diastatic enzymes  $\alpha$ - and  $\beta$ -amylase,  $\alpha$ -glucosidase and limit dextrinase, is important to the success of malting barley cultivars. In addition to being involved in the degradation of solubilised starch,  $\alpha$ -amylase and  $\alpha$ -glucosidase levels have been shown to correlate with the ability of malt extracts to initiate partial degradation (and therefore probably gelatinisation) of starch granules (Sun and Henson, 1990). The levels of  $\alpha$ -amylase and  $\alpha$ -glucosidase in a particular malt may thus be linked to its diastatic power, a malt quality parameter crucial in determining market value, and also to the wort's fermentability (particularly  $\alpha$ -glucosidase).

Barley has one  $\alpha$ -glucosidase gene (Tibbot and Skadsen, 1996) and approximately nine genes encoding  $\alpha$ -amylase, which can be sub-divided into those encoding low-pI isoforms (2-3) and high-pI isoforms (5-6; Chandler et al., 1984). We wanted to investigate the possibility of increasing production of  $\alpha$ -amylase and  $\alpha$ -glucosidase by using transformation to insert extra genes encoding these enzymes. To do this we tested "over-expression" of inserted endogenous cDNAs driven by a native barley high-pI  $\alpha$ -amylase gene promoter, to see if this strategy would increase enzyme levels and if so, whether the diastatic power and/or the extract potential of the malted grain could also be raised. We used an expression cassette derived from a native  $\alpha$ -amylase gene to package and insert cDNAs encoding separately low- and high-pI isoforms of  $\alpha$ -amylase, and  $\alpha$ -glucosidase.

To facilitate proof of concept the first experiments used a low-pI  $\alpha$ -amylase cDNA from Himalaya barley, encoding a very low-pI isoform readily distinguishable from

those of the transformation recipient, Golden Promise (Matthews et al., 1997). After trialing several first generation transformed lines, second generation transgenics were made with a twin T-DNA vector system to enable segregation of SMF plants, and all three cDNAs were inserted separately. As first generation lines showed evidence of somaclonal mutation probably resulting from the transformation process, the SMF lines were then introgressed into the Australian cultivar Sloop. This was to reduce the effect of background mutation and thereby facilitate transgene assessment, and also to facilitate delivery of any improved germplasm to breeding programs.

This paper thus describes outcomes from work done over the last seven years on a project to trial barley transformation breeding, both to explore the overall process, and to improve malting quality by augmentation of malting enzymes. A major focus has also been to produce transgenics which are free of selectable marker genes.

# Materials and Methods

## Plant Material and Transformation

The barley cultivar Golden Promise was used exclusively as transgene recipient in these experiments. Growth conditions for plants used as embryo donors for transformation tissue, and the *Agrobacterium tumefaciens*-mediated transformation procedure, have been described previously (Tingay et al., 1997). Variations used in this study were that scutella were infected with *Agrobacterium* on the day of isolation with no biolistic injury treatment, and co-cultivation was for three days following infection. In the callus induction medium hygromycin replaced bialaphos at 50 mg/l, in the FHG medium at 20 mg/l, and in the rooting medium at 50 mg/l. During selection the division of callus at transfer to fresh media was eliminated. Regenerated T0 plantlets resulting from transformation were grown in phytotron glasshouses (temperature 20/12°C day/night).

## Generation of SMF transformants, and introgression into Sloop

A major focus of transformation was the generation of SMF transformants, which entailed construction and use of a "twin T-DNA" transformation plasmid, and screening of T1 progeny seedlings by PCR for those segregating without the *Hyg* selectable marker gene (Matthews et al., 1999, 2001).

For introgression the recurrent parent, Sloop, was grown in a growth cabinet at 18/12°, and used as the pollen recipient in all crosses. F1 embryos were rescued 19-21 d after crossing, germinated on Gamborgs media (Sigma), and leaf samples analysed for transgene presence by PCR as in the screen for SMF T1 progeny (Matthews et al., 2001). Two transgene-positive plants were then grown in phytotron glasshouses, and one used as pollen donor for the next backcross. When F2 plants were grown, transgene PCR was also used to determine transgene status, firstly from F2 seedlings to discern transgene nulls, secondly for an indication of transgene zygosity in the

remainder (by intensity of the PCRed transgene band) and thirdly (after growth and harvest) for confirmation of homozygosity by assay of 20-23 progeny F3 seedlings.

In the backcrossing program the first cross from a SMF T1 transgenic was termed the outcross; thereafter successive crosses into Sloop were termed backcrosses. Introgression was continued until backcross four (BC4) before growing selfed F2 plants for most lines, however some F2s were also grown at BC1 and/or BC2.

## Transgenes and constructs

The aleurone expression cassette, in which the three cDNAs described here (low- and high-pI  $\alpha$ -amylase, and  $\alpha$ -glucosidase) were separately inserted, was constructed from components of a high-pI  $\alpha$ -amylase gene cloned from the Himalaya cultivar (Jacobsen & Close, 1991). It consisted of 2.2 kb of promoter region fused to the first intron (80 bp), a cDNA and 0.20 kb of terminator region from the same gene, contiguously in that order. The intron, first cDNA used and the terminator were fused together by the technique of splicing by overlap extension (SOE, Horton et al., 1989), then ligated to the promoter section by standard techniques. Inclusion of *SrfI* sites at either end of the first cDNA enabled it to be exchanged with other cDNAs as desired. Constructs made by SOE were always checked by sequencing before proceeding. The very low- and high-pI  $\alpha$ -amylase cDNAs used have been described (Deikman & Jones, 1985; Matthews et al., 1997); that for  $\alpha$ -glucosidase was cloned by RT-PCR from the Himalaya cultivar by homology to a published barley  $\alpha$ -glucosidase sequence (Tibbot & Skadsen, 1996).

A "twin T-DNA" plasmid was created by generating (by SOE) a 1.1 kb fragment containing the T-DNA right and left borders, and inserting it into the *ApaI* site in the polylinker of the transformation vector pWBVec8 (Wang et al., 1998). Different expression cassettes were then ligated into the remaining *HindIII* and *NotI* polylinker sites to create twin T-DNA plasmids containing the hygromycin resistance SM gene in one T-DNA, and the various transgenes in the other (Matthews et al., 1999, 2001).

## Genetic characterisation of transformed lines

Methodology for genomic DNA gel-blot hybridisation, and transgene detection by PCR of crude lysates of leaf samples, has been described (Matthews et al., 2001). PCR of leaf samples from at least 20 progeny seedlings was used to determine homozygosity - for a 3:1 Mendelian ratio this represents a 99% confidence level.

## Quantitation of malting enzymes

To compare production of malting enzymes, plants of introgressed lines at BCF3 generation and homozygous or null for the transgene, were grown in a phytotron greenhouse at 17/12°C in 16 h days. To ensure uniformity of growing conditions, four plants were grown together in a single 19 cm pot, with null and homozygous plants of the same line in two pots adjacent in East-West orientation. Pots were rotated ¼ turn twice a week.

After harvest, 10 plump grains from each of the four plants were combined, de-husked in 50% sulphuric acid for 2 h with slow inversion, washed eight times in distilled water, and cut to remove embryos and distal ends. The half-grains were then sterilised for 30 m in 1% sodium hypochlorite (commercial bleach diluted and adjusted to pH 6.5), washed four times in sterile distilled water and placed on an agarose plate. Each plate was 14 cm in diameter, containing approximately 70 ml of 0.27% agarose buffered with 0.01 M MES at pH 5.9. After 2 d imbibition at 4°C the 40 half-grains were bisected along the suture, and 20 "quarter-grains" placed randomly in each of four 25 ml conical flasks. The flasks contained 2 ml of  $10^{-5}$  M GA<sub>3</sub>,  $10^{-2}$  M CaCl<sub>2</sub>, 150 µg/ml cefotaxime and 50 U/ml nystatin. Incubation occurred under sterile conditions for 1-5 days at 25°C, with samples taken daily for assay of diastatic enzymes.

In some experiments the  $\alpha$ -amylase isoforms in the half-grain media were then fractionated to separate low-pI from high-pI isoforms, by column chromatography over carboxymethyl cellulose (Whatman CM52) in 0.02 M sodium succinate/0.01 M CaCl<sub>2</sub> pH 4.7. Low-pI isoforms eluted in the void volume and high-pI isoforms eluted with a 0.2 M NaCl wash.

For assay of  $\alpha$ -amylase activity a colourimetric determination of soluble starch digestion was used (Chrispeels and Varner, 1967), measuring starch remaining after a timed digestion by change of starch-iodine colour.  $\alpha$ -Glucosidase activity was measured with an adaptation of the method of Sissons and MacGregor (1993), using para-nitrophenyl- $\alpha$ -D-glucoside (pNPG; Sigma) as substrate. 100 µl of 3 mg/ml pNPG, in 0.1 M sodium acetate pH 4.6, was added to 100 µl of sample diluted in the same buffer in a microtitre plate well, incubated for 150 m at 30°C, and the reaction stopped with the addition of 100 µl of 5% Tris buffer (pH not adjusted). Digestion of pNPG was quantified by reading absorbency at 414 nm.

## Isoelectric focusing of $\alpha$ -amylase from individual grains

Individual half-grains, de-husked, de-embryonated and incubated for 2 d on MES plates as described above were then incubated individually in microtitre plate wells with 200 µl of the same GA-containing buffer used for enzyme quantitation, at 25°C in a humid atmosphere. After 3 d incubation, 100 µl samples from each well were heated to 65°C for 10 m, assayed for  $\alpha$ -amylase and diluted to equivalent activity before loading a sample of each grain's media on an individual lane of an IEF gel. The gel used was an Ampholine PAG plate, pH 4.0-6.5 (Pharmacia), run on an LKB 2117 Multiphor apparatus. Gels were pre-focused for >2 h at 500 V, run for 2 h at 500 V followed by 30 m at 1100 V. For enzyme detection, gels were incubated for 30 m in a 2% soluble starch (Merck #1257) solution followed by incubation in 7.2 mM KI, 1 mM I<sub>2</sub> in 50 mM HCl.

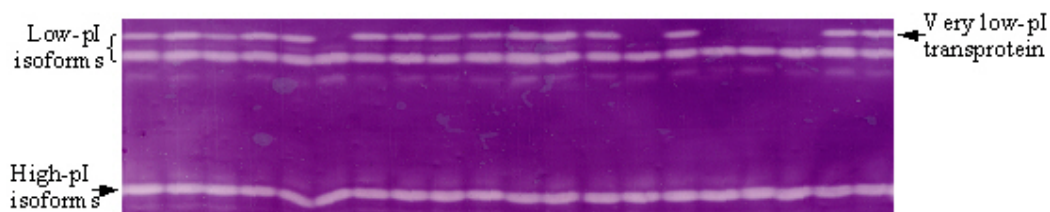
# Results

## Molecular characterisation of transgenic lines

Characterisation by DNA-blot hybridisation (Southern analysis) of T1 plants of most of the SMF transgenic lines generated in this study has been described (Matthews et al., 2001), and T0 plants of first generation lines were analysed in a similar way. All SMF lines contained a single transgene insert as inferred from segregation ratios. The copy number of transgene inserts was determined, and absence of the selectable marker gene also confirmed, by cutting genomic DNA with two different restriction enzymes and using probes for the 3' end of the expression cassette or the marker gene. While some first generation lines showed multiple transgene copy numbers and /or inserts, all SMF lines contained a single transgene insert, and all inserts had a transgene copy number of one except the line LPI4, which showed a copy number of two on DNA gel-blot analysis.

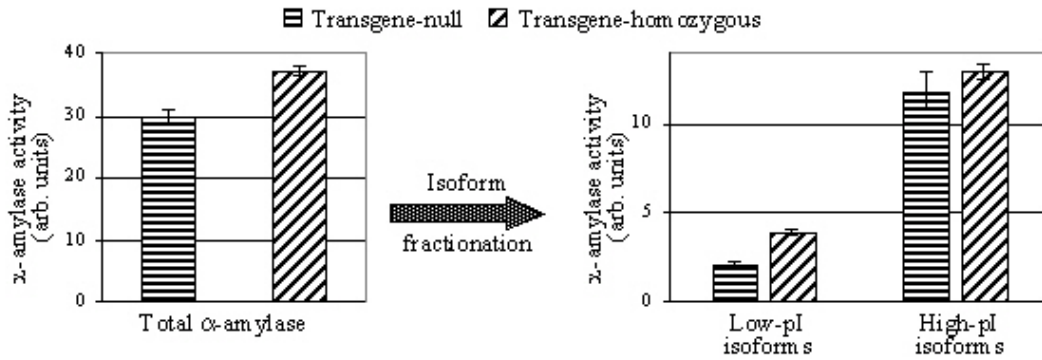
## Studies with first generation transformed lines

Before the twin T-DNA technology was used to produce SMF transformants, a number of transgenic Golden Promise lines were made using the aleurone expression cassette in pWBVec5 (Wang et al., 1998), a vector utilising bialaphos resistance as a selectable marker. Several lines containing inserts of the low- and high-pI  $\alpha$ -amylase cDNAs were generated and tested for transgene function. Figure 1 shows one such test, where grains from a T0 plant (T1 grain), which contained two transgene copies by DNA-blot analysis (data not shown), were pre-treated and incubated as described prior to iso-electric focusing. The gel, with lanes from 20 half-grains, clearly shows expression of the very low-pI  $\alpha$ -amylase transprotein in 15 of the 20 grains.



**Figure 1:** Isoelectric focusing of  $\alpha$ -amylase from 20 T1 grains of a first generation transformed line, containing 2 copies of the very low-pI  $\alpha$ -amylase transgene.

Several T2 plants of another first generation line, transformed with the very low-pI  $\alpha$ -amylase and containing 4-5 transgene copies by DNA-blot analysis, were grown. Transgene-null and -homozygous T3 grain was tested for  $\alpha$ -amylase production as described, except using 100 grains comprising 25 from each of two plants of each zygosity, in a total of ten flasks. After incubation for 32 h in GA<sub>3</sub> media,  $\alpha$ -amylase levels were measured, then enzyme isoforms were separated and re-assayed after fractionation over carboxymethyl cellulose. Figure 2 shows the results of this experiment, which indicates an increase of around 20% in total amylase produced, and an increase of around 100% in the low-pI isoforms, when separated by chromatography.

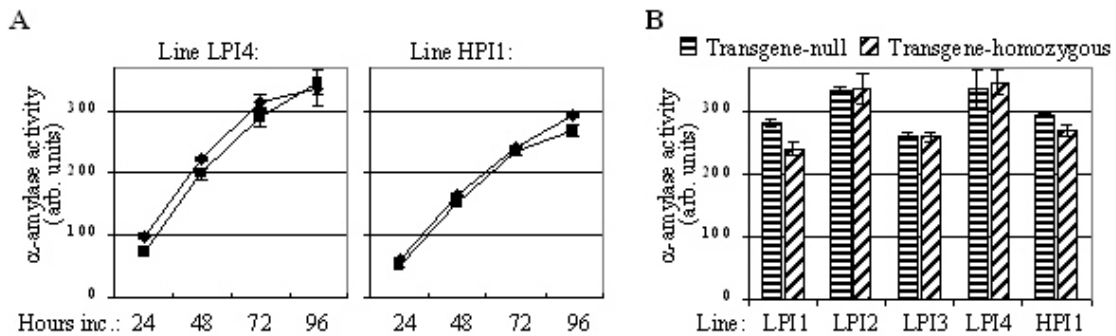


**Figure 2:** Production of  $\alpha$ -amylase by T3 grains of a first generation transformed line, containing 4-5 copies of the very low-pI  $\alpha$ -amylase transgene, and fractionation of isoforms.

## Production of $\alpha$ -amylase from transgenic grain

For quantifying  $\alpha$ -amylase five of the most advanced SMF lines were selected at varying stages of introgression, comprising four with the low-pI  $\alpha$ -amylase transgene at BC4 (lines LPI1-4 in Matthews et al., 2001) and one with the high-pI  $\alpha$ -amylase transgene at BC2 (Line HPI1). Zygosity of BCF2 plants was determined, and BCF3 plants were grown as described, followed by GA<sub>3</sub> incubation of BCF4 grains for four days.

Figure 3A shows the 0-96 h time-course of  $\alpha$ -amylase production for null- and homozygous-transgene grain from the low- and high-pI  $\alpha$ -amylase-transformed lines LPI4 and HPI1, with each time-point value expressed as mean  $\pm$  SEM from the four flasks. These are shown as examples; the other three lines were not different in character to these, and there was no significant and consistent trend from transgene-null to -homozygous grain across the five lines. Figure 3B shows the values of  $\alpha$ -amylase production observed at 96 h incubation for all five lines. It is apparent that no consistent significant difference between values for null- and homozygous-transgene grain was produced. Differences in the 96 h levels achieved between the various lines may be due to genetic divergence, but are more likely due to small variations between experiments or in growth conditions (for example the de-husking procedure was found to strongly influence  $\alpha$ -amylase production). Two lines show significantly lower  $\alpha$ -amylase production in transgene-homozygous than in transgene-null grain, namely lines LPI1 and HPI1. Whether this is an indication of a true deleterious effect of the transgene insert, or results from unknown environmental and/or genetic effects, remains to be determined.

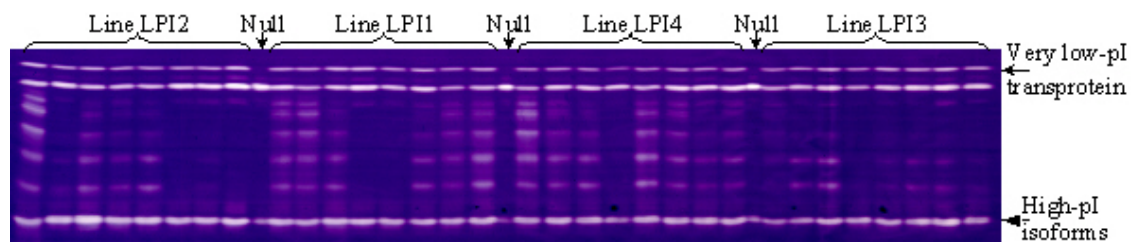


**Figure 3:** Activity (arbitrary OD units) of  $\alpha$ -amylase produced from null- and homozygous-transgene BCF3 plants. **A:** Time-courses of  $\alpha$ -amylase production by null- (◆) and homozygous-transgene (■) grain incubated for 96 h at 25C. **B:**  $\alpha$ -Amylase production from the five transgenic lines at 96 h.

The 96 h incubation media from three of these five experiments was further analysed by chromatography over carboxymethylcellulose, to separate and quantify the contribution of each isoform class to the total  $\alpha$ -amylase present in grain media. However, again no consistent, significant difference between transgene-null and -homozygous grain was observed (data not shown).

## Isoelectric focusing of $\alpha$ -amylase isoforms from individual grains

To test whether the lack of transgene influence on  $\alpha$ -amylase production reported above was due to high frequency transgene silencing, 8 grains from one of the homozygous BC4F3 plants of each of the four low-pI  $\alpha$ -amylase transformed lines were pre-treated and GA-incubated for 3 d as described, and media samples run on an IEF gel. This is shown in Figure 4.



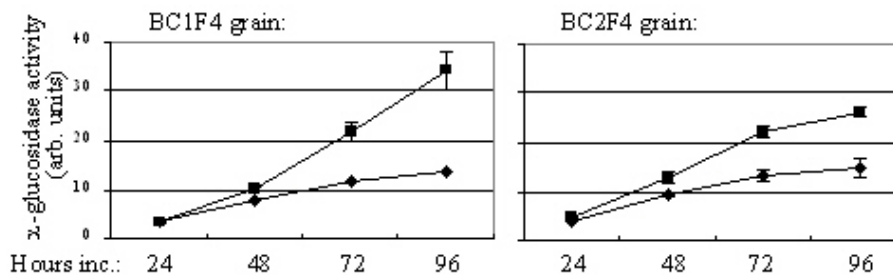
**Figure 4:** Isoelectric focusing of  $\alpha$ -amylase isoforms from the four transgenic lines at BC4F3. Eight grains of each line are shown, separated by  $\frac{1}{2}$ -width lanes from a transgene-null control. Faint bands between the two  $\alpha$ -amylase isoform groups are  $\beta$ -amylase incompletely degraded by the 65°C treatment.

It is apparent from the uniform presence of the transgenic low-pI  $\alpha$ -amylase isoform band (the "very low-pI" isoform) that no high-frequency silencing of the transgene has occurred. However, the transprotein band in line LPI4, which contains two transgene copies (compared to one copy in the other lines; Matthews et al., 2001) is

not noticeably more intense than that in the other three lines. This implies either that one of the two transgene copies in LPI4 is inactive (possible, but not apparent on DNA gel-blot analysis), or that some factor other than gene copy number is limiting transprotein production.

## Production of $\alpha$ -glucosidase from transgenic grain

Plants of the  $\alpha$ -glucosidase-transformed line AG1 were grown and pre-treated in the same way as for the  $\alpha$ -amylase-transformed lines, except that BCF3 plants were grown at both the BC1 and BC2 generations. Half-grains from these plants were pre-treated and incubated in the same GA-medium at 25°C for 96 h (120 h for the BC1 grain), and their media sampled and assayed for  $\alpha$ -glucosidase daily. Figure 5 shows the results of these two time-course experiments.



**Figure 5:** Time-courses of activity (arbitrary OD units) of  $\alpha$ -glucosidase produced from null- (◆) and homozygous-transgene (■) BCF3 plants of line AG1.

It is apparent from these time-courses that the presence of the  $\alpha$ -glucosidase transgene has a dramatic effect on the production of  $\alpha$ -glucosidase in these conditions, more than doubling the enzyme activity in media from the BC1 grain, and causing a large increase in production from BC2 grain. Whether the lower increase in BC2 compared to BC1 grain is due to the additional backcross is doubtful, and uncertainty also arises from the low number of  $\alpha$ -glucosidase transformed lines observed (one). However it seems likely that this transgene-linked effect is genuine, and a direct result of the augmentation of the endogenous  $\alpha$ -glucosidase gene with this transgene cassette.

## Discussion

The transformation breeding of barley is an emerging technology with a large potential for improving the performance of both the crop and its grain. To explore strategies and processes required to help realise this potential was one of this project's most significant objectives, and in terms of this aim it has been quite successful. Thus we have pioneered a new and improved technique of barley transformation, developed and implemented a strategy for creating SMF transgenic lines, and used introgression of SMF transgenes into an elite barley cultivar to circumvent deleterious somaclonal mutations and facilitate delivery to industry. These strategies could be used to develop

transgenic lines containing transgenes with potential for improvement of barley cultivars, or introduce novel traits to expand the end uses of the grain.

The results of our attempts to improve barley malting quality are more mixed. To increase enzyme production in a transgenic plant by increasing the gene copy number for that enzyme obviously requires that gene copy number is limiting in enzyme expression. The results presented here, although preliminary, indicate that this is probably not the case for barley  $\alpha$ -amylase, but may be so for  $\alpha$ -glucosidase. While disappointing in terms of possible barley improvement, this result matches what is known about copy numbers for the endogenous barley genes - there appears to be about nine  $\alpha$ -amylase genes (three low-pI and six high-pI; Chandler et al., 1984), but only one  $\alpha$ -glucosidase gene (Tibbot and Skadsen, 1996). Thus, it is hardly surprising that addition of an extra  $\alpha$ -amylase gene makes little or no difference, but addition of an  $\alpha$ -glucosidase gene - an effective doubling of gene copy number - makes a considerable difference. This is supported by the results from examination of the second low-pI  $\alpha$ -amylase transformed first generation line (Figure 2), which showed multiple transgene copies (4-5 by DNA-blot analysis compared to one or two for the SMF lines) and a considerable increase in low-pI  $\alpha$ -amylase production from transgene-null to -homozygous grain.

Be that as it may, the potential for barley improvement from either of the transgenes tested remains to be determined, as the assessment conducted here is quite divorced from the reality of the malting situation. In malting or germination, the exposure of aleurone cells to GA is much reduced compared to these experiments, as is the degree of  $\alpha$ -amylase gene induction. Indeed, in standard malts of Schooner barley, low-pI  $\alpha$ -amylase isoforms are almost completely absent, as the induction of these genes is delayed compared to high-pI genes during germination (Karrer et al, 1991). Whether the small amounts of very low-pI transprotein expressed during malting make a detectable difference to crucial malting quality parameters, when larger samples of transgenic grain are micromalted, remains to be seen.

To test this, the six introgressed, transgenic lines used in these experiments were also grown in a small-scale field trial in the spring and summer of 2000, and the harvest of transgenic grain is currently undergoing analysis by micromalting. The results of these tests are awaited with interest.

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