Transgene Segregation to Obtain Selectable Marker-Free Transformed Barley

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Abstract

A technique for the generation of selectable marker-free (SMF) transgenic barley lines has been tested and adopted for the routine insertion of malting quality and other transgenes into barley. An *Agrobacterium* transformation vector containing two T-DNA regions was constructed, with a selectable marker gene encoding hygromycin resistance in one T-DNA and various "genes of interest" (GOI) in the other.
GOIs encoding barley low-isoelectric point (pI) α-amylase, high-pI α-amylase and α-glucosidase have been used in the system. An initial PCR screen was performed on plantlets leaving tissue culture to detect those which carry the GOI as well as the selectable marker, while screening for lines that segregate SMF plants was performed on shoots of T_1 grain.

On present results it appears that 60-70% of transformants (ie: T_0 plants containing the hygromycin resistance gene) carry the GOI, and of these 20-30% segregate SMF T_1 progeny. So far 16 SMF lines have been generated, and all appear to have one to three GOI copies, generally inserted at a single locus.

Introduction

There is today increasing public, industry and scientific concern regarding the proliferation of antibiotic and herbicide resistance genes in the environment, particularly those genes used as selectable markers in plant genetic transformation. Regardless of the likelihood of transfer of these genes to other organisms, whether in the field or in the gut, the need for the generation of SMF transgenic lines in the genetic engineering of crops is becoming ever more apparent. While alternative, more benign marker genes may become available, there now are several ways in which the selectable marker (SM) gene can be removed from engineered lines after transformation.

One method for doing this when using transformation with *Agrobacterium tumefaciens* involves the creation of first generation (T_0) plants which have the two transgenes, the SM and GOI, co-inserted independently in the recipient genome, enabling segregation of SMF T_1 progeny in the following generation. The production of the desired independent co-insertion events was accomplished at a reasonably high frequency in tobacco and rice by Komari et al. (1996), using transformation with a super binary *Agrobacterium* transformation vector containing two well-separated T-DNA regions, one containing the SM gene and the other the GOI. Analysis of T_1 seedlings revealed plants containing only the GOI in about 25% of transformed lines, which applied to our present efficiency of barley transformation represents an acceptable loss in output from our transformation program in order to obtain the desired SMF lines.

To adapt this system to the genes and transformation vectors we are presently using in barley transformation, we added another T-DNA region to the cereal transformation vector pWBVec8 (Wang et al., 1998), and then inserted different GOIs into this T-DNA. Although the two T-DNAs in the resulting construct were almost contiguous, we still obtained a comparable frequency of independent co-insertion in the transformation events mediated by them. The selection of SMF plants was aided by the adaptation of a methodology enabling facilitated analysis of T_1 seedlings, using PCR to simultaneously detect the presence or absence of both transgenes.

Materials and Methods

Plant Material

The cultivar Golden Promise was used for all experiments, as described by Tingay et al. (1997). Growth of T_0 and T_1 plants was in a phytotron greenhouse. Rescued embryos were grown on Gamborg's medium at 22-24°C.

DNA Constructs

The expression cassette was constructed using the method of splicing by overlap extension (SOE; Horton et al., 1989; Olsen, 1992), joining the first intron from the barley high-pI α-amylase gene to a cDNA open reading frame (ORF) and the terminator from the same gene. This DNA amplimer was ligated to the high-pI α-amylase promoter by standard cloning techniques, and the sequence checked. Inclusion of *Srf*I sites at either end of the ORF enabled it to be exchanged with other cDNA amplimers as desired. The α-amylase cDNAs used have been described (Deikman & Jones, 1985; Matthews et al., 1997); that for α-glucosidase was cloned by PCR from the Himalya cultivar by homology to a published barley α-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 *Apa*I site in the pWBVec8 polylinker. Different expression cassettes were then ligated into the remaining *Hin*dIII and *Not*I polylinker sites to create twin T-DNA plasmids containing the hygromycin resistance SM gene in one T-DNA, and the various GOIs in the other.

Barley Transformation

Transformation utilized immature scutella starting material and was as described by Tingay et al. (1997), with the following modifications:

Scutella were infected with Agrobacterium tumefaciens on the day of isolation with no biolistic injury treatment. 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.

Analysis of T_1 Progeny

Approximately three weeks after anthesis grain from T_0 plants was harvested, surface sterilized and the embryos excised. These were grown on Gamborg's medium for 1-2 weeks until seedlings were 2-10 cm long, and small leaf sections (2-4 mm) excised for PCR analysis. The leaf sections were placed in 0.5 ml plastic tubes to which was added 50 μl of the TPS buffer of Thomson and Henry (1995), containing 100 mM Tris pH 9.5, 1 M KCl, 10 mM EDTA, together with four 1.5 – 2 mm glass balls. The tubes were vigorously agitated by placing 6-12 in a 55 mm x 42 mm plastic jar fixed to a vortexer and vortexing on high for 1-2 min. After a brief centrifugation 0.5 μl of supernatant was used in a 10 μl PCR reaction having 10 mM Tris pH 8.2, 3.5 mM $MgCl_2$ and 0.5 units of Taq polymerase. Four primers were used, one pair generating a 917 bp product from the hygromycin resistance gene, the other pair generating both a 758 bp product from the endogenous high-pI α-amylase gene (as a reaction control) and a 678 bp product from the transgene (80 bp shorter due to intron positioning). Reactions were analysed on 1.8% agarose electrophoresis gels.

Results and Discussion

Transformation of Golden Promise barley with a twin T-DNA binary transformation plasmid, derived from pWBVec8 and containing the GOI encoding the very low-pI α-amylase in the "new" T-DNA, was initiated to test the effectiveness and practicability of the system. The first experiments were also used to maximize efficiency of the screening procedures, and involved analysis of 31 transformed lines obtained at a transformation efficiency of approximately 5%. Initially T_0 plants were screened by Southern analysis to determine whether or not they carried the GOI in addition to the hygromycin resistance SM gene. Later this test was replaced by PCR of a leaf fragment at a much earlier stage, prior to planting out a plantlet in soil from the rooting media. Of the 31 starting T_0 lines, we found 19 which contained the gene of interest, and these were grown on to produce T_1 grain, embryo rescues being performed about three weeks after anthesis. Embryos were germinated on Gamborg's media, and when seedlings were 1 – 2 weeks old they were tested again for presence of the two transgenes.

Initial experiments at this stage used a leaf section to test for hygromycin resistance by the method of Wang and Waterhouse (1997); those seedlings testing sensitive to hygromycin were then tested for presence of the GOI by PCR analysis of small-scale DNA preparations. Following adaptation of the disruption lysate technique mentioned above, the leaf test for hygromycin resistance was abandoned in favor of performing one multiplex PCR analysis on all seedlings – the resulting presence/absence of the three bands then indicates the following:
Whether the PCR reaction was successful (758 bp band from endogenous α-amylase gene).
Whether the SM transgene is present (917 bp band from hygromycin resistance gene).
Whether the GOI transgene is present (678 bp band).

Routinely we analysed 50 – 150 T_1 seedlings from each T_0 line, looking firstly for segregants showing no SM PCR product, then among these for those showing GOI amplimer. We saw no obviously non-Mendelian transgene segregation ratios. Among no-SM progeny, segregation ratios implied either one SM locus (three to one ratio), two loci (fifteen to one ratio) or ≥3 loci (≥ 63 to 1 ratio). Thus among the 19 lines tested we saw seven lines with one SM locus, six with two loci and six with three or more loci. Six of the 19 lines gave rise to T_1 progeny showing presence of the GOI but not the SM transgene – the desired SMF transgenic plants. These plants were later analysed by Southern hybridization to confirm the transgene status, probing with sections from the two transgenes (SM and GOI).

Meanwhile barley transformation has been continuing with twin T-DNA binary plasmids containing the other two GOIs – encoding a high-pI α-amylase and an α-glucosidase. So far 30 lines containing the α-glucosidase GOI and 12 lines containing the high-pI α-amylase GOI have been tested for segregation of T_1 progeny. Seven of the α-glucosidase lines produced SMF T_1 progeny, as did three of the high-pI α-amylase lines. Segregation and PCR data for one of the three high-pI lines strongly suggest that it contains at least two copies of the GOI located at two distinct chromosomal loci, both distinct from the locus at which the SM gene inserted.

So far, 61 GOI-containing lines have been analysed for segregation of transgenes, yielding 16 (26%) SMF lines. In T_0 plantlets, 66% of transformed lines (carrying the SM gene) also carry the GOI. When combined with the proportion of these lines that yield SMF progeny, 17% of transformed lines in this system have independent co-insertion of the two T-DNAs and produce SMF progeny.

This figure is lower than the >25% obtained in rice by Komari et al. (1996); its acceptability depends on how many SMF lines are ultimately required for a transformation breeding program. It may be that the larger sections of intervening DNA sequence between T-DNAs in the "super-binary" transformation vectors employed by these workers (several kilobases as opposed to about 850 bp) favor independent co-insertion. If so, a re-engineering of the twin T-DNA vectors used in this study may improve results. However, the twin T-DNA system has been shown here to be capable of producing barley transformants free of the undesirable SM genes, and should be of value in barley transformation breeding.

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