RESEARCH

# A Single Backcross Effectively Eliminates Agronomic and Quality Alterations Caused by Somaclonal Variation in Transgenic Barley

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**ABSTRACT**

Transgenic crops have proven commercial utility but are created using processes known to produce undesirable variability known as somaclonal variation. This reduces the utility of transgenic germplasm to the plant breeder and complicates assessments of transgene-encoded phenotypes. Backcrossing transgenes into a wild-type genome is one solution, but producing near-isogenic lines requires a lengthy and resource-intensive process of multiple crosses. However, an abbreviated breeding scheme involving a single backcross to the wild-type parent used to produce a transgenic line, which would replace 75% of the variant alleles, should produce transgenic lines with improved performance. Comparisons were made of 'Conlon' barley (*Hordeum vulgare* L.), primary transgenic lines derived from Conlon, and lines derived from single backcrosses of primary transgenic lines to Conlon. The primary transgenic lines were different from Conlon for many agronomic and malting characteristics. Most of the backcross-derived lines did not differ significantly from Conlon for most agronomic characteristics. The backcross-derived lines were also similar to Conlon for malting quality traits but showed more differences than for agronomic characteristics. Differences between lines encoding *TRI101* versus lines encoding *PDR5* suggested that *PDR5* insertion or expression may have affected malting quality. It is concluded that a single backcross is an effective, rapid, and inexpensive method for creating superior transgenic lines.

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**Abbreviations:** BC, backcross; FAN, free amino nitrogen; PCR, polymerase chain reaction.

Nonsexual introduction of recombinant DNA has proven utility for the creation of novel and useful genetic variability. Several transgenic crops have widespread commercial success—transgenic maize (*Zea mays* L.) encoding herbicide resistance, for example, accounted for 26% of maize planted in the United States in 2006 (USDA, National Agricultural Statistics Service: http://usda.mannlib.cornell.edu/usda/nass/Acre//2000s/2006/Acre-06-30-2006.pdf; verified 21 Jan. 2008). However, the initial creation of transgenic cereal genotypes relies heavily on in vitro techniques that have long been known to generate somaclonal variation (Larkin and Scowcroft, 1981). Somaclonal variation can result in heritable changes to both qualitative and quantitative traits (Kaeppler et al., 1998; Kaeppler et al., 2000) attributable to genetic and epigenetic changes at numerous locations, including point mutations, methylation changes, insertions and deletions, the activation of transposable elements, and gross chromosomal rearrangements (Kaeppler et al., 1998; Kaeppler et al., 2000).

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The productivity and quality characteristics of modern cultivars are the result of a lengthy, incremental process of selection for particular allelic combinations at multiple loci. The introduction of random somaclonal variation would be expected, and has been demonstrated, to have primarily negative consequences. In barley (*Hordeum vulgare* L.), simple passage through tissue culture has been shown to significantly reduce yield and malting quality in a majority of derived lines (Bregitzer and Poulson, 1995; Bregitzer et al., 1995), although lines with negligible changes can also be recovered (see Bregitzer et al. [2002] and references therein). The process of transformation (in barley as well as in other cereals) imposes additional stress that exacerbates somaclonal variation (Bregitzer et al., 1998; Choi et al., 2000a; Choi et al., 2000b; Choi et al., 2001; Horvath et al., 2001; Schuh et al., 1993), and transgenic lines derived by self-pollination of the original transgenic event typically have significant and sometimes severe (greater than 50%) yield reductions.

In the context of mainstream agricultural production, even slight performance reductions are generally unacceptable. Furthermore, somaclonal variation complicates evaluations of transgene-encoded phenotypes by introducing multiple unrelated sources of variability that are confounded with the effects of transgene insertion and expression. Transgenic germplasm thus will be of most value for genetic investigations and for plant breeding if somaclonal variation is minimized.

The generation of somaclonal variation can be reduced by technical improvements to the transformation process (Bregitzer et al., 2002), but a more generally applicable approach may be simply to use backcrossing to eliminate variant alleles in favor of wild-type alleles present in the recurrent parent. A lengthy process involving many backcrossing cycles would be necessary to completely eliminate variant alleles, but even a short process involving two crosses to the original cultivar used to produce the transgenic parent should produce significant improvements. This would theoretically eliminate 75% of the variant alleles that differentiate the transgenic line from its wild-type parent, thus facilitating meaningful comparisons. Furthermore, it is possible that the reduction of epigenetic alterations may be more rapid than predicted based on quantitative genetic models. A particularly intriguing example is that of a dwarf mutant of rice (*Oryza sativa*)—putatively associated with altered methylation patterns—that was stably transmitted by self-pollination but which could not be recovered on outcrossing to a parent of normal height (Oono, 1985).

Several studies have reported performance improvements via crosses and backcrosses of transgenic parents (Horvath et al., 2001; Shao et al., 2006), but these studies did not use the original wild-type cultivar as the recurrent parent. This study was conducted to determine the utility of a simple and rapid breeding scheme—a single backcross of transgenic barley plants to the original parent used in their production—for the recovery of recurrent parent phenotype as measured by variation in agronomic and malting quality characteristics.

# MATERIALS AND METHODS

## Line Development

For this report, the performance of the malting cultivar Conlon was compared to 43 lines derived from four transgenic events that were produced in the background of Conlon using published methods (Manoharan and Dahleen, 2002).

Eight lines (primary transgenic lines), two derived from each of four transgenic events, were derived directly from transgenic plants via self-pollination. The two lines from each event were designated as lines "A" and "B" and represented random selections among $T_0$ plants arising from different sectors of the callus derived from that event. The data in this report were collected from field tests of $T_{2:4}$ and $T_{2:5}$ lines in 2005 and 2006, respectively. Seed for these tests was produced from field increases at Aberdeen, ID, of $T_{2:3}$ and $T_{2:4}$ seed in 2004 and 2005, respectively. $T_{2:3}$ seed was produced in the greenhouse at Aberdeen, ID, from small populations (5 to 10 plants) in the winter of 2003–2004. Prior generation advance had been conducted via single seed descent in the greenhouse in Fargo, ND. These lines were produced by cotransformation with pAHC25 (Christensen and Quail, 1996), which includes the selectable marker *bar*, in combination with either maize-ubiquitin-driven *TRI101* (primary transgenic lines pUBR1-II and pUBR1-III) (Manoharan et al., 2006) or *PDR5* (primary transgenic lines pUBR2-I and pUBR2-II). *TRI101,* pUBR1, and *PDR5* have been previously described (Balzi et al., 1994; Kimura et al., 1998; Okubara et al., 2002). pUBR2 is identical to pUBR1 except for the substitution of *PDR5* for *TRI101.*

The remaining 35 lines were derivatives of a single backcross (BC) to Conlon (Conlon/$T_3$//Conlon), and the data in this report were collected from field tests of $BC_1F_{2:5}$ and $BC_1F_{2:6}$ lines in 2005 and 2006, respectively. Seed for these tests was produced from field increases at Aberdeen, ID, of $BC_1F_{2:4}$ and $BC_1F_{2:5}$ seed in 2004 and 2005, respectively. $BC_1F_{2:4}$ seed was produced in the greenhouse at Aberdeen, ID, from small $BC_1F_{2:3}$ populations (8–16 plants) in the winter of 2003–2004. Ten transgenic and 7 null (nontransgenic) segregant lines were derived from crosses to pUBR1 lines, and 11 transgenic and 7 null segregant lines were derived from crosses to pUBR2 lines. Since both null segregant and transgenic backcross-derived populations include somaclonal variation inherited from the primary transgenic parent, comparisons of null-segregant and transgenic line performance enable estimation of phenotypic variability induced by transgene insertion and/or expression. For the purposes of this report, a primary transgenic parent and the backcross-derived lines derived from that parent are referred to as a family. A complete listing of transgenic lines and family relationships can be found in Tables 1 and 2.

The backcross-derived families were produced from each of the eight primary transgenic lines via selection solely for the presence or absence of transgenic loci among small populations. All selections and generation advances were conducted in the greenhouse at Aberdeen, ID, through the $BC_1F_{2:3}$. For each family, a single $F_1$ plant was backcrossed to Conlon, and hemizygous $BC_1F_1$ progeny plants were identified among small populations (15 to 25 plants) based on *bar*-encoded resistance to glufosi-

**Table 1. Agronomic performance of 'Conlon' barley, transgenic parents expressing *TRI101*, and transgenic and null (N) backcross ($BC_1$) derivative lines.**

| Transgenic parent | Line | Heading | *p* | Plant height | *p* | Test weight | *p* | Grain yield | *p* | Plump kernels | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | d after 1 Jan. | | cm | | kg $m^{-3}$ | | g | | % | |
| | Conlon | 174.1 | | 82 | | 663 | | 594 | | 81.4 | |
| pUBR1-IIA | pUBR1-IIA | 176.5 | <0.0001 | 76 | 0.03 | 644 | <0.0001 | 400 | <0.0001 | 77.6 | <0.0001 |
| | 2-13 | 174.9 | 0.35 | 85 | 1.00 | 666 | 1.00 | 564 | 0.73 | 80.4 | 0.42 |
| | 3-12 (N) | 174.7 | 0.60 | 83 | 1.00 | 656 | 0.34 | 564 | 0.75 | 81.3 | 0.96 |
| | 3-9 | 174.4 | 0.91 | 83 | 1.00 | 660 | 0.84 | 594 | 0.97 | 80.8 | 0.70 |
| | 4-13 (N) | 175.8 | 0.003 | 84 | 1.00 | 660 | 0.89 | 560 | 0.69 | 80.5 | 0.49 |
| Mean of derived lines | | 174.9 | | 84 | | 661 | | 570 | | 80.7 | |
| (% of Conlon, parent) | | (101.4) | | (92.7) | | (97.1) | | (67.3) | | (95.3) | |
| (% of Conlon, derived lines) | | (100.5) | | (101.9) | | (99.7) | | (96.0) | | (99.2) | |
| pUBR1-IIB | pUBR1-IIB | 176.4 | <0.0001 | 78 | 0.22 | 640 | <0.0001 | 401 | <0.0001 | 78.6 | 0.0001 |
| | 2-5 | 175.5 | 0.02 | 84 | 1.00 | 658 | 0.56 | 523 | 0.07 | 81.0 | 0.88 |
| | 2-7 | 174.6 | 0.76 | 84 | 1.00 | 657 | 0.46 | 577 | 0.87 | 81.2 | 0.94 |
| | 3-4 | 174.3 | 0.95 | 83 | 0.99 | 660 | 0.87 | 554 | 0.64 | 80.9 | 0.83 |
| | 5-14 (N) | 174.5 | 0.79 | 84 | 1.00 | 663 | 0.98 | 552 | 0.65 | 80.6 | 0.62 |
| Mean of derived lines | | 174.7 | | 83 | | 659 | | 551 | | 81.0 | |
| (% of Conlon, parent) | | (101.3) | | (94.8) | | (96.6) | | (67.4) | | (96.5) | |
| (% of Conlon, derived lines) | | (100.3) | | (101.2) | | (99.5) | | (92.7) | | (99.4) | |
| pUBR1-IIIA | pUBR1-IIIA | 176.4 | <0.0001 | 81 | 0.92 | 642 | <0.0001 | 391 | <0.0001 | 77.9 | <0.0001 |
| | 4-6 (N) | 174.5 | 0.81 | 83 | 1.00 | 659 | 0.71 | 598 | 0.99 | 81.4 | 0.98 |
| | 6-17 | 174.7 | 0.64 | 84 | 1.00 | 659 | 0.78 | 586 | 0.94 | 81.2 | 0.93 |
| | 6-4 | 174.9 | 0.34 | 83 | 0.99 | 657 | 0.44 | 527 | 0.19 | 80.9 | 0.81 |
| | 8-15 (N) | 174.9 | 0.38 | 83 | 1.00 | 662 | 0.97 | 582 | 0.94 | 81.4 | 0.98 |
| Mean of derived lines | | 174.7 | | 83 | | 659 | | 573 | | 81.2 | |
| (% of Conlon, parent) | | (101.3) | | (98.8) | | (96.9) | | (65.7) | | (95.7) | |
| (% of Conlon, derived lines) | | (100.3) | | (101.3) | | (99.5) | | (96.4) | | (99.7) | |
| pUBR1-IIIB | pUBR1-IIIB | 177.3 | <0.0001 | 75 | 0.01 | 636 | <0.0001 | 338 | <0.0001 | 76.1 | |
| | 2-15 | 174.4 | 0.91 | 83 | 1.00 | 664 | 0.99 | 568 | 0.84 | 80.7 | 0.64 |
| | 3-11 | 175.0 | 0.29 | 84 | 1.00 | 661 | 0.94 | 545 | 0.55 | 81.6 | 0.99 |
| | 4-12 (N) | 174.8 | 0.41 | 84 | 1.00 | 656 | 0.25 | 540 | 0.35 | 81.8 | 1.00 |
| | 5-7 (N) | 175.3 | 0.04 | 84 | 1.00 | 661 | 0.93 | 508 | 0.07 | 81.8 | 1.00 |
| | 8-3 | 175.3 | 0.06 | 83 | 0.99 | 667 | 1.00 | 524 | 0.13 | 81.6 | 0.99 |
| Mean of derived lines | | 175.0 | | 84 | | 662 | | 537 | | 81.5 | |
| (% of Conlon, parent) | | (101.8) | | (91.3) | | (96.0) | | (56.9) | | (93.5) | |
| (% of Conlon, derived lines) | | (100.5) | | (101.5) | | (99.9) | | (90.3) | | (100.1) | |

nate-ammonium damage. Glufosinate-ammonium resistance was assessed on seedlings (three- to five-leaf stage) by applying glufosinate-ammonium as a 0.5 g $kg^{-1}$ solution to small sections of two leaves of each tested plant. Tween-20 was included as a surfactant (1% v/v). From 4 to 14 resistant $BC_1F_1$ plants were advanced to $BC_1F_2$ via single seed descent, and $BC_1F_2$ populations of 18 to 30 plants were screened for glufosinate-ammonium resistance as described above. A total of 10 $BC_1F_2$ plants (including 2 that were susceptible to glufosinate-ammonium damage and presumably homozygous for the absence of transgenic loci) were selected at random. Homozygous lines were identified, and hemizygous lines were eliminated based on $BC_1F_{2:3}$ progeny tests for resistance or susceptibility to glufosinate-ammonium damage. Genetic variability within each family was maximized by deriving selections from multiple $BC_1F_1$ parents. Final selections included lines tracing to 2 to 5 $BC_1F_1$ parents (see Tables 1 and 2). The line designations denote the $BC_1F_1$ parent and the $BC_1F_2$ parent; for example, pUBR1-IIA 2-13 was from $BC_1F_1$ plant no. 2 and $BC_1F_2$ plant no. 13. Phenotypic assessments were confirmed by polymerase chain reaction (PCR) verification of the presence or absence of either *TRI101* or *PDR5*. Polymerase chain reaction assays were performed using a forward primer hybridizing to a sequence in the ubiquitin intron (pAHC17F: TTT AGC CCT GCC TTC ATA CG) in combination with either the reverse primer TRI101R (GGA GAC TGA TTT GGG TGT AGA TCG) or the reverse primer PDR5R (GTC AGA GTC CTT GCC AGT TTT TGG).

The expression of the selectable marker, *bar*, in all the backcross-derived lines, and by inference in all the primary transgenic lines, was demonstrated by the success of the selection process described above. In addition, the expression of *TRI101* has been demonstrated in the primary transgenic parents and both of two backcross-derived lines tested via northern and immunoblot assays (Manoharan et al., 2006). Northern assays

Table 2. Agronomic performance of 'Conlon' barley, transgenic parents expressing *PDR5*, and transgenic and null (N) backcross ($BC_1$) derivative lines.

| Transgenic parent | Line | Heading | *p* | Plant height | *p* | Test weight | *p* | Grain yield | *p* | Plump kernels | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | d after 1 Jan. | | cm | | kg $m^{-3}$ | | g | | % | |
| | Conlon | 174.1 | | 82 | | 663 | | 594 | | 81.4 | |
| pUBR2-IA | pUBR2-IA | 176.1 | <0.0001 | 77.3 | 0.05 | 647 | 0.0001 | 385 | <0.0001 | 80.5 | 0.47 |
| | 5-4 | 174.6 | 0.74 | 82.6 | 0.97 | 657 | 0.35 | 532 | 0.17 | 81.5 | 0.99 |
| | 6-22 | 174.1 | 0.98 | 81.4 | 0.86 | 658 | 0.57 | 537 | 0.28 | 81.1 | 0.88 |
| | 7-11 (N) | 174.0 | 0.99 | 81.5 | 0.87 | 653 | 0.04 | 552 | 0.58 | 81.4 | 0.97 |
| | 9-3 | 174.1 | 0.98 | 81.9 | 0.92 | 660 | 0.77 | 561 | 0.68 | 81.6 | 0.99 |
| Mean of derived lines | | 174.2 | | 81.9 | | 657 | | 545 | | 81.4 | |
| (% of Conlon, parent) | | (101.1) | | (93.4) | | (97.6) | | (64.7) | | (98.9) | |
| (% of Conlon, derived lines) | | (100.0) | | (98.9) | | (99.1) | | (91.7) | | (99.9) | |
| pUBR2-IB | pUBR2-IB | 176.1 | 0.0002 | 76.9 | 0.03 | 649 | 0.0012 | 370 | <0.0001 | 79.9 | 0.07 |
| | 2-19 | 174.2 | 0.97 | 82.6 | 0.97 | 656 | 0.22 | 554 | 0.68 | 82.0 | 1.00 |
| | 2-3 | 174.4 | 0.86 | 82.3 | 0.96 | 657 | 0.33 | 566 | 0.75 | 82.1 | 1.00 |
| | 3-5 | 174.2 | 0.96 | 82.4 | 0.97 | 656 | 0.25 | 547 | 0.47 | 81.7 | 1.00 |
| | 3-7 (N) | 174.2 | 0.97 | 85.8 | 1.00 | 666 | 1.00 | 576 | 0.89 | 81.8 | 1.00 |
| | 4-4 (N) | 174.2 | 0.97 | 82.8 | 0.98 | 660 | 0.78 | 557 | 0.73 | 81.6 | 0.99 |
| Mean of derived lines | | 174.3 | | 83.2 | | 659 | | 560 | | 81.9 | |
| (% of Conlon, parent) | | (101.1) | | (92.9) | | (97.9) | | (62.2) | | (98.1) | |
| (% of Conlon, derived lines) | | (100.1) | | (100.5) | | (99.4) | | (94.2) | | (100.5) | |
| pUBR2-IIA | pUBR2-IIA | 174.2 | 0.96 | 80.6 | 0.71 | 653 | 0.04 | 500 | 0.03 | 81.2 | 0.94 |
| | 1-2 (N) | 174.3 | 0.92 | 82.9 | 0.98 | 663 | 0.98 | 572 | 0.82 | 81.5 | 0.98 |
| | 1-8 | 174.1 | 0.99 | 82.3 | 0.96 | 662 | 0.96 | 548 | 0.46 | 81.9 | 1.00 |
| | 3-2 (N) | 174.6 | 0.74 | 84.4 | 1.00 | 661 | 0.88 | 571 | 0.87 | 81.4 | 0.98 |
| | 3-7 | 174.3 | 0.93 | 84.6 | 1.00 | 658 | 0.45 | 587 | 0.96 | 81.5 | 0.98 |
| | 3-8A | 174.2 | 0.97 | 83.8 | 1.00 | 664 | 0.99 | 609 | 1.00 | 81.6 | 0.99 |
| Mean of derived lines | | 174.3 | | 83.6 | | 661 | | 577 | | 81.6 | |
| (% of Conlon, parent) | | (100.1) | | (97.4) | | (98.5) | | (84.1) | | (99.8) | |
| (% of Conlon, derived lines) | | (100.1) | | (101.0) | | (99.8) | | (97.1) | | (100.2) | |
| pUBR2-IIB | pUBR2-IIB | 174.3 | 0.95 | 77.1 | 0.04 | 651 | 0.007 | 494 | 0.01 | 81.2 | 0.94 |
| | 3-8 (N) | 174.9 | 0.38 | 86.7 | 1.00 | 664 | 1.00 | 576 | 0.88 | 81.5 | 0.99 |
| | 5-5 | 174.2 | 0.98 | 81.9 | 0.93 | 656 | 0.23 | 543 | 0.35 | 81.6 | 0.99 |
| | 6-15 | 174.4 | 0.91 | 84.3 | 1.00 | 663 | 0.98 | 567 | 0.72 | 81.5 | 0.98 |
| | 6-9 (N) | 174.3 | 0.92 | 81.6 | 0.89 | 660 | 0.74 | 563 | 0.67 | 81.5 | 0.99 |
| Mean of derived lines | | 174.4 | | 83.6 | | 661 | | 562 | | 81.5 | |
| (% of Conlon, parent) | | (100.1) | | (93.2) | | (98.2) | | (83.1) | | (99.7) | |
| (% of Conlon, derived lines) | | (100.2) | | (101.0) | | (99.7) | | (94.6) | | (100.1) | |

have shown *PDR5* transcription in all primary transgenic lines (Dahleen and Manoharan, unpublished data).

## Agronomic Analyses

Field trials were conducted in 2005 and 2006 at Aberdeen, ID, and in 2006 at Langdon, ND. Supplemental irrigation was used at both sites. The experimental design was a randomized complete block, with six replicates at Aberdeen and five replicates at Langdon. Each plot consisted of a single 3-m row. Planting was done with four-row headrow drills. At Langdon, rows were planted on 30.5-cm centers. At Aberdeen, rows were planted on 35.6-cm centers, and each plot was separated by a single row of wheat (*T. aestivum* L.). Seeding rates were 4 g per plot at Langdon and 8 g per plot at Aberdeen. Heading date (visual estimate of the date of 50% spike extrusion from the boot) and plant height (to the top of spikes not including awns) were recorded. Plots were harvested using single-row binders and small-plot threshers. Following harvest, seed samples were cleaned and analyzed for yield, test weight, and percentage plump kernels (defined as the percentage of kernels retained on a 2.38 × 19.1-mm sieve).

## Malt Analyses

These analyses were conducted on the primary transgenic parents and on two transgenic backcross-derived lines from each family. Limited resources prevented analysis of grain from all plots, so at both locations two samples, each a composite of equal amount of grain from two replicates, were analyzed for each tested line.

Barley protein was determined with a Leco Nitrogen Analyzer model FP528 (Leco Corporation, St. Joseph, MI). Barley moisture was determined according to American Society of Brewing Chemists method Barley 5AC (ASBC, 1992). Thin kernels, passing through a sieve with 1.98 × 19.00-mm slotted openings,

were removed before malting. Micromalting was performed in duplicate on each barley sample according to our standard method (Karababa et al., 1993). Time required to reach 45% steep-out moisture was first determined by pilot-steeping a 10-g sample. Steeping of 80-g samples was at 16°C with a 1 h air rest included for each 12 h of steeping. The steep water was aerated 6 min $h^{-1}$. Germination was for 4 d at 16°C and ~95% relative humidity. Samples were turned daily by hand to prevent matting, and sample weight was adjusted to 45% moisture with distilled water. Kilning was conducted in a forced-air laboratory kiln. Total kiln time was 24 h, during which temperatures were ramped from 49 to 85°C. Rootlets were removed from the kilned malt before analysis.

Malt moisture, extract, wort soluble protein, wort color, free amino nitrogen (FAN), wort viscosity, and wort β-glucan were determined by ASBC methods Malt-3, Malt-4, Wort-3, Malt-5A, Wort-9, Wort-12, Wort-13, and Wort-18, respectively (ASBC, 1992). The ratio of soluble/total protein was calculated using the value for barley protein. Alpha-amylase and diastatic power were determined by a ferricyanide-reducing sugar method using flow injection, as previously described (Karababa et al., 1993).

### Data Analysis

Data were analyzed by SAS v. 8.0 Proc GLM (1999, SAS Institute Inc., Cary, NC). The models used to examine line performance data included the following sources of variability: environment (Aberdeen 2005, Aberdeen 2006, and Langdon 2006), replicate-within-environment, line, and line × environment. Replicate-within-environment, environment, and line × environment were considered random, and line × environment was used as the error term. The data were discarded from one replicate at Aberdeen in 2005 because of extreme soil variability and from 25 plots at Langdon that suffered damage from herbicide drift. The data are therefore presented as least-squares means.

The performance of primary transgenic or backcross-derived line performance was compared to Conlon performance using one-tailed Dunnett's comparisons for traits showing a clear, unidirectional change in the primary transgenic parents relative to Conlon (most traits); otherwise, they were analyzed with two-tailed Dunnett's tests. Since the objective of this study was to determine whether somaclonal variation present in the primary transgenic lines remained in their backcross-derived progeny, it was important to avoid a type II error (failure to reject the null hypothesis $H_0$: mean of Conlon = mean of derived line), and a conservative $p$ value of >0.2 was used as the basis for declaring that means were equal.

## RESULTS AND DISCUSSION

### Agronomic Performance

Visual observations were made at least biweekly at the Aberdeen location in 2005 and 2006. Germination, stand establishment, and growth for the first several weeks did not differ among lines to any detectable degree. From the point of the stem elongation phase through maturity, certain plots became distinguishable based on having plants that had fewer tillers, developed slightly slower, often appeared to be shorter, and generally had a less vigorous appearance. Just before heading, these plots were noted (without consulting the field book to avoid bias), and then the identity of the line in each of these plots was determined. None were backcross-derived lines. Without exception, all were primary transgenic lines. Almost all plots containing pUBR1-IIA, pUBR1-IIB, pUBR1-IIIA, or pUBR1-IIIB; many of the plots containing pUBR2-IA and pUBR2-IB; and almost none of the plots containing pUBR2-IIA and pUBR2-IIB were noted as being less vigorous. The same lines with qualitative, visual reductions in vigor were noted in both 2005 and 2006, suggesting that generation advance had no significant effect on the reduction of variability. This is consistent with previous comparisons of $T_2$ and $T_4$ barley lines (Bregitzer et al., 1998).

Quantification of heading date, plant height, grain yield per plot, test weight, and plump kernel percentage from Aberdeen and Langdon showed results that were generally consistent with the visual observations conducted at Aberdeen (Tables 1 and 2). The primary transgenic lines pUBR2-IIA and -IIB showed the fewest instances of significant differences from Conlon, and the magnitude of significant differences was generally less than that seen for other lines. All primary transgenic lines except for pUBR2-IIA and -IIB headed later than Conlon. These two lines, plus pUBR2-IA, also did not differ from Conlon for the percentage of plump kernels. Plant height was less than Conlon except for pUBR1-IIB, pUBR1-IIIA, and pUBR2-IIA. All primary transgenic lines had lower grain yield and test weight than Conlon, but the smallest reductions were seen for pUBR2-IIA and -IIB. The trait showing the greatest reduction in the primary transgenic lines was yield, which ranged from 57 to 84% (with a mean of 69%) of that recorded for Conlon. This is also consistent with our previous assessments of tissue culture–derived and transgenic line performance (Bregitzer and Poulson, 1995; Bregitzer et al., 1998; Bregitzer et al., 2006). It is not surprising that yield would be more affected than other agronomic traits, since it is arguably dependent on the expression of a greater number of genes than most other traits.

In contrast to the performance of the primary transgenic lines, the backcross-derived lines showed few instances of significant differences from Conlon, even when taking the conservative approach to avoiding a type II error by declaring significant differences only at a $p$ value of 0.2 or less (Tables 1 and 2). Relative to Conlon, four lines headed later, none were shorter, five yielded less, one had lower test weight, and none had a lower percentage of plump kernels. Despite the general lack of significant differences from Conlon, careful examination of the data suggested an overall trend for lower performance yields. There was a nonnormal distribution of individual backcross-derived line means about the mean of Conlon, and mean yields of the eight backcross-derived families ranged from 90 to 97% of Conlon (with an overall mean

Table 3. Malting characteristics of 'Conlon' barley, transgenic parents expressing *TRI101*, and transgenic backcross ($BC_1$) derivative lines.

| Transgenic parent | Line | Barley protein | *p* | Soluble protein | *p* | Soluble/total protein | *p* | Free amino nitrogen | *p* | Malt extract | *p* | Diastatic power | *p* | α-amylase | *p* | β-glucan | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | | % | | | | | | % | | | | | | μg $g^{-1}$ | |
| | Conlon | 12.2 | | 5.0 | | 0.41 | | 211 | | 79.7 | | 116 | | 65 | | 260 | |
| pUBR1-IIA | pUBR1-IIA | 13.2 | 0.001 | 5.0 | 0.90 | 0.38 | 1.00 | 221 | 0.61 | 76.3 | <0.0001 | 105 | 1.00 | 64 | 1.00 | 226 | 0.72 |
| | 2-13 | 12.8 | 0.10 | 5.2 | 0.64 | 0.41 | 0.96 | 220 | 0.65 | 78.4 | 0.01 | 118 | 0.91 | 67 | 1.00 | 209 | 0.50 |
| | 3-9 | 12.4 | 0.78 | 5.0 | 0.96 | 0.40 | 0.99 | 204 | 1.00 | 78.7 | 0.08 | 113 | 0.99 | 63 | 1.00 | 245 | 0.89 |
| Mean of derived lines | | 12.6 | | 5.1 | | 0.40 | | 212 | | 78.5 | | 116 | | 65 | | 227 | |
| (% of Conlon, parent) | | (108.5) | | (101.7) | | (93.7) | | (104.7) | | (95.8) | | (90.6) | | (98.6) | | (86.9) | |
| (% of Conlon, derived lines) | | (103.2) | | (102.5) | | (99.2) | | (100.4) | | (98.6) | | (99.6) | | (99.4) | | (87.3) | |
| pUBR1-IIB | pUBR1-IIB | 13.4 | <0.0001 | 5.2 | 0.59 | 0.39 | 1.00 | 224 | 0.42 | 76.1 | <0.0001 | 112 | 0.99 | 62 | 0.78 | 236 | 0.82 |
| | 2-5 | 12.4 | 0.67 | 5.1 | 0.87 | 0.41 | 0.95 | 217 | 0.80 | 78.9 | 0.25 | 122 | 0.73 | 69 | 0.65 | 224 | 0.70 |
| | 2-7 | 12.5 | 0.50 | 5.0 | 0.91 | 0.40 | 0.98 | 227 | 0.27 | 78.5 | 0.04 | 118 | 0.92 | 69 | 0.88 | 198 | 0.37 |
| Mean of derived lines | | 12.5 | | 5.0 | | 0.41 | | 222 | | 78.7 | | 120 | | 69 | | 211 | |
| (% of Conlon, parent) | | (110.3) | | (105.2) | | (95.7) | | (106.3) | | (95.6) | | (96.6) | | (94.6) | | (90.6) | |
| (% of Conlon, derived lines) | | (102.4) | | (101.7) | | (99.5) | | (105.3) | | (98.8) | | (103.6) | | (105.4) | | (81.3) | |
| pUBR1-IIIA | pUBR1-IIIA | 13.1 | 0.006 | 5.2 | 0.57 | 0.40 | 0.99 | 231 | 0.14 | 76.5 | <0.0001 | 122 | 0.72 | 65 | 1.00 | 221 | 0.67 |
| | 6-17 | 12.1 | 0.97 | 5.1 | 0.86 | 0.42 | 0.83 | 212 | 0.95 | 79.6 | 0.94 | 131 | 0.18 | 69 | 0.80 | 218 | 0.62 |
| | 6-4 | 12.5 | 0.50 | 5.1 | 0.87 | 0.40 | 0.97 | 223 | 0.50 | 79.1 | 0.46 | 121 | 0.81 | 64 | 1.00 | 242 | 0.87 |
| Mean of derived lines | | 12.3 | | 5.1 | | 0.40 | | 217 | | 79.4 | | 126 | | 66 | | 230 | |
| (% of Conlon, parent) | | (107.3) | | (105.6) | | (98.4) | | (109.2) | | (96.1) | | (105.3) | | (100.0) | | (85.2) | |
| (% of Conlon, derived lines) | | (101.2) | | (102.1) | | (101.0) | | (103.0) | | (99.6) | | (108.6) | | (101.7) | | (88.5) | |
| pUBR1-IIIB | pUBR1-IIIB | 13.2 | 0.001 | 5.1 | 0.85 | 0.39 | 1.00 | 219 | 0.72 | 76.7 | <0.0001 | 119 | 0.90 | 61 | 0.58 | 247 | 0.91 |
| | 2-15 | 12.3 | 0.87 | 5.2 | 0.68 | 0.42 | 0.75 | 212 | 0.95 | 79.8 | 0.99 | 130 | 0.22 | 67 | 1.00 | 186 | 0.23 |
| | 3-11 | 12.3 | 0.81 | 5.1 | 0.81 | 0.41 | 0.90 | 214 | 0.90 | 78.7 | 0.09 | 129 | 0.32 | 68 | 0.89 | 217 | 0.61 |
| Mean of derived lines | | 12.3 | | 5.1 | | 0.42 | | 213 | | 79.3 | | 129 | | 68 | | 202 | |
| (% of Conlon, parent) | | (108.2) | | (102.9) | | (94.9) | | (103.7) | | (96.2) | | (102.5) | | (93.7) | | (95.1) | |
| (% of Conlon, derived lines) | | (101.2) | | (103.7) | | (102.6) | | (100.9) | | (99.5) | | (111.5) | | (103.8) | | (77.6) | |

of 94%). Furthermore, perusal of the data presented in Tables 1 and 2 reveals rough correlation between the performance of transgenic parents and their backcross-derived lines. Consistent with such a correlation, the highest-yielding primary transgenic line (pUBR2-IIA) produced the best-yielding family of backcross-derived lines, and the lowest-yielding primary transgenic line (pUBR1-IIIB) produced the lowest-yielding family of backcross-derived lines. Thus, rather than being an artifact of sampling error, this trend may be viewed as the expected result if it is assumed that the yield depression in the primary transgenic parents is caused by alterations at many loci, each having small, equivalent, additive, and negative effects. If it is further assumed that the recovery of recurrent parent performance is proportional to the degree to which the recurrent parent genome replaces that of the primary transgenic parent, then the predicted yield loss after a single backcross would be 25% of the original yield loss relative to Conlon. Although these assumptions are undoubtedly simplistic, it is interesting that they predict a mean yield for backcross-derived lines of 548 g $plot^{-1}$, very similar to the observed performance of 559 g $plot^{-1}$.

Comparisons of the data in Tables 1 and 2 for backcross-derived line performance indicate no association of the presence or absence (null segregants) of transgenes on any measure of agronomic performance. Independent statistical analyses that excluded data for Conlon and the primary transgenic lines also failed to detect differences between transgenic and null segregant lines for grain yield (model and data not shown). These results suggest that the main source of variability in the primary transgenic lines relative to Conlon was somaclonal variation rather than

effects of transgene insertion or expression. This is consistent with previous studies of transgenic barley and wheat (Barro et al., 2002; Bregitzer et al., 1998; Bregitzer et al., 2006; Zhou et al., 2003), although other studies have detected phenotypic alterations in transgenic plants as a result of transgene insertion or expression (Horvath et al., 2001; Lee et al., 2003).

## Malt Analyses

The barley protein content of all primary transgenic lines showed significant increases relative to Conlon (Tables 3 and 4). Soluble protein levels, the ratio of soluble to total protein, and FAN levels were significantly elevated in pUBR2-IA and pUBR2--IB. The FAN level of pUBR1-IIIA showed a slight increase.

Increased protein levels typically are associated with decreased malt extract levels and increased enzymatic activities (α-amylase and diastatic power). Malt extract levels showed a downward trend for the primary transgenic lines, although the decreases did not reach the 0.2 probability level for pUBR2-IIA and pUBR2-IIB. However, diastatic power showed significant increases only for the pUBR2 primary transgenic lines, suggesting that factors other than increased protein were involved in variability for enzymatic activity. A similar trend toward increased activity was seen for α-amylase but was significant only for pUBR2-IIB.

β-glucan levels also showed distinct differences between the two groups of primary transgenic lines. All of the pUBR2 lines had significantly lower levels than Conlon, while none of the pUBR1 lines were significantly different from Conlon.

The backcross-derived families showed general improvements for characteristics that were changed in their primary transgenic parents, but there were several important exceptions. All families showed a trend

**Table 4. Malting characteristics of 'Conlon' barley, transgenic parents expressing *PDR5*, and transgenic backcross ($BC_1$) derivative lines.**

| Transgenic parent | Line | Barley protein | *p* | Soluble protein | *p* | Soluble/total protein | *p* | Free amino nitrogen | *p* | Malt extract | *p* | Diastatic power | *p* | α-amylase | *p* | β-glucan | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | | % | | | | | | g k | | | | | | μg g$^{-1}$ | |
| | Conlon | 12.2 | | 5.0 | | 0.41 | | 211 | | 79.7 | | 116 | | 65 | | 260 | |
| pUBR2-IA | pUBR2-IA | 13.7 | <0.0001 | 6.1 | 0.0002 | 0.44 | 0.17 | 249 | 0.001 | 77.7 | <0.0001 | 139 | 0.02 | 70 | 0.44 | 171 | 0.12 |
| | 5-4 | 13.1 | 0.003 | 5.6 | 0.07 | 0.42 | 0.70 | 232 | 0.11 | 79.4 | 0.80 | 139 | 0.02 | 73 | 0.03 | 170 | 0.11 |
| | 6-22 | 12.0 | 0.99 | 5.2 | 0.68 | 0.43 | 0.48 | 215 | 0.87 | 79.7 | 0.97 | 129 | 0.29 | 71 | 0.28 | 138 | 0.02 |
| Mean of derived lines | | 12.6 | | 5.4 | | 0.40 | | 224 | | 79.5 | | 134 | | 72 | | 154 | |
| (% of Conlon, parent) | | (112.5) | | (122.5) | | (109.1) | | (117.9) | | (97.5) | | (119.9) | | (107.0) | | (66.0) | |
| (% of Conlon, derived lines) | | (103.3) | | (108.4) | | (105.0) | | (106.0) | | (99.8) | | (115.6) | | (109.8) | | (59.3) | |
| pUBR2-IB | pUBR2-IB | 13.9 | <0.0001 | 6.2 | <0.0001 | 0.44 | 0.16 | 244 | 0.004 | 77.6 | <0.0001 | 144 | 0.004 | 69 | 0.62 | 153 | 0.04 |
| | 2-19 | 13.0 | 0.01 | 5.0 | 0.92 | 0.39 | 1.00 | 195 | 1.00 | 79.3 | 0.73 | 138 | 0.05 | 68 | 0.98 | 225 | 0.71 |
| | 2-3 | 12.7 | 0.16 | 5.2 | 0.71 | 0.41 | 0.96 | 206 | 0.99 | 79.5 | 0.90 | 139 | 0.02 | 67 | 1.00 | 186 | 0.23 |
| Mean of derived lines | | 12.8 | | 5.1 | | 0.40 | | 200 | | 79.4 | | 138 | | 68 | | 205 | |
| (% of Conlon, parent) | | (113.8) | | (124.2) | | (109.3) | | (115.4) | | (97.3) | | (123.8) | | (106.1) | | (59.0) | |
| (% of Conlon, derived lines) | | (105.5) | | (102.5) | | (97.4) | | (94.9) | | (99.7) | | (119.2) | | (103.6) | | (79.0) | |
| pUBR2-IIA | pUBR2-IIA | 13.1 | 0.005 | 5.5 | 0.12 | 0.42 | 0.78 | 217 | 0.82 | 79.0 | 0.34 | 146 | 0.002 | 70 | 0.49 | 142 | 0.02 |
| | 1-8 | 12.4 | 0.70 | 5.1 | 0.77 | 0.41 | 0.90 | 207 | 0.99 | 80.0 | 1.00 | 138 | 0.03 | 72 | 0.03 | 174 | 0.14 |
| | 3-7 | 12.6 | 0.31 | 5.4 | 0.24 | 0.43 | 0.59 | 214 | 0.90 | 80.1 | 1.00 | 154 | <0.0001 | 74 | 0.005 | 165 | 0.09 |
| Mean of derived lines | | 12.5 | | 5.3 | | 0.42 | | 211 | | 80.0 | | 146 | | 73 | | 170 | |
| (% of Conlon, parent) | | (107.4) | | (110.5) | | (103.1) | | (102.6) | | (99.2) | | (125.9) | | (106.7) | | (54.5) | |
| (% of Conlon, derived lines) | | (102.7) | | (106.0) | | (103.3) | | (99.8) | | (100.5) | | (126.0) | | (112.0) | | (65.3) | |
| pUBR2-IIB | pUBR2-IIB | 12.8 | 0.06 | 5.4 | 0.27 | 0.42 | 0.79 | 221 | 0.62 | 79.0 | 0.36 | 156 | <0.0001 | 75 | 0.004 | 137 | 0.01 |
| | 5-5 | 12.6 | 0.25 | 5.0 | 0.93 | 0.40 | 1.00 | 212 | 0.94 | 79.6 | 0.92 | 148 | 0.001 | 72 | 0.11 | 168 | 0.10 |
| | 6-15 | 12.6 | 0.37 | 5.2 | 0.53 | 0.42 | 0.84 | 226 | 0.31 | 79.7 | 0.97 | 143 | 0.004 | 72 | 0.04 | 115 | 0.003 |
| Mean of derived lines | | 12.6 | | 5.1 | | 0.41 | | 219 | | 79.6 | | 146 | | 72 | | 142 | |
| (% of Conlon, parent) | | (105.3) | | (108.3) | | (103.1) | | (104.7) | | (99.2) | | (134.6) | | (114.0) | | (52.8) | |
| (% of Conlon, derived lines) | | (103.5) | | (103.2) | | (99.8) | | (103.9) | | (99.9) | | (125.7) | | (110.2) | | (54.4) | |

toward greater similarity to Conlon for barley protein, soluble protein, the ratio of soluble to total protein, and FAN, but there were several instances of large and significant differences, primarily in backcross-derived lines derived from the primary transgenic lines, that showed the greatest differences from Conlon (pUBR2-IA, -IAB). Malt extract showed a similar trend of improvement in the backcross-derived lines, again with instances of significant differences from Conlon being associated with primary transgenic lines having the greatest reductions relative to Conlon.

Diastatic power and the levels of α-amylase and β-glucan, in particular, deviated from the trend toward recovery of Conlon levels for the backcross-derived lines. Data from the pUBR1 families, which showed no significant changes for these traits, have little informative value, although it is interesting to note that for diastatic power, one could argue that there is a trend toward higher levels (and thus greater deviations from Conlon) in the backcross-derived lines than in the primary transgenic parents. Of much more significance, however, is the general lack of recovery of Conlon levels of performance for these traits in the families derived from the transformation events pUBR2-I and pUBR2-II, which is evidenced both by a majority of lines showing significant differences from Conlon and the lack of a clear trend toward Conlon performance when compared with the performance of the primary transgenic lines. Consideration of the contrasting family performance (*PDR5* vs. *TRI101*), the general failure to recover recurrent parent phenotype, and the lack of variability between the *PDR5*-containing families derived from different transgenic events suggests that the expression of *PDR5* may influence these traits. Examination of the malting performance of null segregant lines derived from backcrosses to *PDR5* lines would be necessary to make a definitive conclusion regarding *PDR5*-induced variability. However, the differences in phenotypic effects are consistent with the functions of the two genes: *PDR5* encodes a transport protein with a wide variety of targets (Golin et al., 2007), while *TRI101* encodes an acetyltransferase with just a few known substrates (Kimura et al., 1998).

## CONCLUSIONS

This study demonstrates an effective strategy for producing transgenic barley lines in which somaclonal variability for agronomic performance is minimized. Backcrossing effectively restored much or all of the performance of the original parent (Conlon), whereas transgenic lines advanced by self-pollination showed multiple differences from Conlon. The predictable recovery of recurrent parent phenotype suggests that the epigenetic changes present in the primary transgenic parents were stable and subject to Mendelian patterns of inheritance. Malting quality showed a similar trend, although the lack of data for null segregants in the pUBR2 does not allow distinguishing the effects of somaclonal variation from those of transgene expression or insertion. Overall, when there was no evidence of a phenotypic effect of a transgenic locus, the results were consistent with the expectation that a single backcross would replace 75% of the donor parent genome and produce progeny lines that are more similar to the recurrent parent than to the donor parent.

The process described in this report is relatively simple and rapid and requires minimal resources. Phenotypic selection among large populations in the field was not necessary—selection was based exclusively on the presence of the transgene among very small populations in the greenhouse. When combined with improved in vitro techniques that generate less somaclonal variation and enable transformation of elite cultivars (Bregitzer et al., 2002; Dahleen and Bregitzer, 2002; Manoharan and Dahleen, 2002; Zhang et al., 1999), superior transgenic barley germplasm can be produced. The reduction of somaclonal variation in transgenic lines facilitates accurate determinations of the phenotypic effect(s) and commercial value of transgene expression by enabling more nearly isogenic comparisons with the nontransformed parent. Furthermore, the phenotype conferred by the transgenic trait can be more realistically evaluated if it expressed a wild-type background, free of any pleiotropic interactions with variant alleles that may have arisen by somaclonal variation during the transformation process.

## References

American Society of Brewing Chemists. 1992. Methods of analysis. 8th ed. American Society of Brewing Chemists, St. Paul, MN.

Balzi, E., M. Wang, S. Leterme, L. Van Dyck, and A. Goffeau. 1994. PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. J. Biol. Chem. 269:2206–2214.

Barro, F., P. Barcelo, P.A. Lazzeri, P.R. Shewry, A. Martin, and J. Ballesteros. 2002. Field evaluation and agronomic performance of transgenic wheat. Theor. Appl. Genet. 105:980–984.

Bregitzer, P., A.E. Blechl, D. Fiedler, J. Lin, P. Sebesta, J.F. De Soto, O. Chicaiza, and J. Dubcovsky. 2006. Changes in high molecular weight glutenin subunit composition can be genetically engineered without affecting wheat agronomic performance. Crop Sci. 46:1553–1563.

Bregitzer, P., S.E. Halbert, and P.G. Lemaux. 1998. Somaclonal variation in the progeny of transgenic barley. Theor. Appl. Genet. 96:421–425.

Bregitzer, P., and M. Poulson. 1995. Agronomic performance of barley lines derived from tissue culture. Crop Sci. 35:1144–1148.

Bregitzer, P., M. Poulson, and B.L. Jones. 1995. Malting quality of barley lines derived from tissue culture. Cereal Chem. 72:433–435.

Bregitzer, P., S.B. Zhang, M.J. Cho, and P.G. Lemaux. 2002. Reduced somaclonal variation in barley is associated with culturing highly differentiated, meristematic tissues. Crop Sci. 42:1303–1308.

Choi, H.W., P.G. Lemaux, and M. Cho. 2000a. High frequency of cytogenetic aberration in transgenic oat (*Avena sativa* L.) plants. Plant Sci. 156:85–94 [erratum: 160(4):761–772].

Choi, H.W., P.G. Lemaux, and M.J. Cho. 2000b. Increased chromosomal variation in transgenic versus nontransgenic barley (*Hordeum vulgare* L.) plants. Crop Sci. 40:524–533.

Choi, H.W., P.G. Lemaux, and M.J. Cho. 2001. Selection and osmotic treatment exacerbate cytological aberrations in transformed barley (*Hordeum vulgare*). J. Plant Physiol. 158:935–943.

Christensen, A.H., and P.H. Quail. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgen. Res. 5:213–218.

Dahleen, L.S., and P. Bregitzer. 2002. An improved media system for high regeneration rates from barley immature embryo-derived callus cultures of commercial cultivars. Crop Sci. 42:934–938.

Golin, J., S.V. Ambudkar, and L. May. 2007. The yeast Pdr5p multidrug transporter: How does it recognize so many substrates? Biochem. Biophys. Res. Commun. 356:1–5.

Horvath, H., L.G. Jensen, O.T. Wong, E. Kohl, S.E. Ullrich, J. Cochran, C.G. Kannangara, and D. Von Wettstein. 2001. Stability of transgene expression, field performance and recombination breeding of transformed barley lines. Theor. Appl. Genet. 102:1–11.

Kaeppler, S.M., H.F. Kaeppler, and Y. Rhee. 2000. Epigenetic aspects of somaclonal variation in plants. Plant Mol. Biol. 43:179–188.

Kaeppler, S.M., R.L. Phillips, and P. Olhoft. 1998. Molecular basis of heritable tissue culture-induced variation in plants. p. 465–484. *In* D.S.B.J.S.M. Jain and B.S. Ahloowalia (ed.) Somaclonal variation and induced mutations in crop improvement. Current Plant Science and Biotechnology in Agriculture, vol. 32. Kluwer Academic, Dordrecht, The Netherlands.

Karababa, E., P.B. Schwarz, and R.D. Horsley. 1993. Effect of kiln schedule on micromalt quality parameters. Am. Soc. Brew. Chem. 51:163–167.

Kimura, M., I. Kaneko, M. Komiyama, A. Takatsuki, H. Koshino, K. Yoneyama, and I. Yamaguchi. 1998. Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins: Cloning and characterization of Tri101. J. Biol. Chem. 273:1654–1661.

Larkin, P.J., and W.R. Scowcroft. 1981. Somaclonal variation: A novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197–214.

Lee, S.B., H.B. Kwon, S.J. Kwon, S.C. Park, M.J. Jeong, S.E. Han, M.O. Byun, and H. Daniell. 2003. Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. Mol. Breed. 11:1–13.

Manoharan, M., and L.S. Dahleen. 2002. Genetic transformation of the commercial barley (*Hordeum vulgare* L.) cultivar Conlon by particle bombardment of callus. Plant Cell Rep. 21:76–80.

Manoharan, M., L.S. Dahleen, T.M. Hohn, S.M. Neate, X.H. Yu, N.J. Alexander, S.P. McCormick, P. Bregitzer, P.B. Schwarz, and R.D. Horsley. 2006. Expression of 3-OH trichothecene acetyltransferase in barley (*Hordeum vulgare* L.) and effects on deoxynivalenol. Plant Sci. 171:699–706.

Okubara, P.A., A.E. Blechl, S.P. McCormick, N.J. Alexander, R. Dill-Macky, and T.M. Hohn. 2002. Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. Theor. Appl. Genet. 106:74–83.

Oono, K. 1985. Putative homozygous mutations in regenerated plants of rice. Mol. Gen. Genet. 198:377–384.

Schuh, W., M.R. Nelson, D.M. Bigelow, T.V. Orum, C.E. Orth, P.T. Lynch, P.S. Eyles, N.W. Blackhall, J. Jones, E.C. Cocking, and M.R. Davey. 1993. The phenotypic characterisation of R2 generation transgenic rice plants under field conditions. Plant Sci. 89:69–79.

Shao, Q.M., M.C. Rush, M.S. Wu, D.E. Groth, M.S. Kang, and S.D. Linscombe. 2006. Transgene transfer to United States commercial rice cultivars via conventional breeding techniques. J. Crop Improv. 16:49–65.

Zhang, S., M.J. Cho, T. Koprek, R. Yun, P. Bregitzer, and P.G. Lemaux. 1999. Genetic transformation of commercial cultivars of oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) using in vitro shoot meristematic cultures derived from germinated seedlings. Plant Cell Rep. 18:959–966.

Zhou, H., J.D. Berg, S.E. Blank, C.A. Chay, G. Chen, S.R. Eskelsen, J.E. Fry, S. Hoi, T. Hu, P.J. Isakson, M.B. Lawton, S.G. Metz, C.B. Rempel, D.K. Ryerson, A.P. Sansone, A.L. Shook, R.J. Starke, J.M. Tichota, and S.A. Valenti. 2003. Field efficacy assessment of transgenic Roundup Ready wheat. Crop Sci. 43:1072–1075.