Theor Appl Genet (1998) 96:421–425

P. Bregitzer · S. E. Halbert · P. G. Lemaux

Somaclonal variation in the progeny of transgenic barley

Received: 26 June 1997 / Accepted: 31 October 1997

Abstract Somaclonal variation (SCV) in transgenic plants may slow the incorporation of introduced genes into commercially competitive cultivars. Somaclonal variation in transgenic barley (*Hordeum vulgare* L.) was assessed in one experiment by comparing the agronomic characteristics of 44 segregating transgenic lines in the T_2 generation to their non-transformed parent ('Golden Promise'). A second experiment examined the agronomic characteristics of seven transgenic-derived, null (non-transgenic) segregant lines in the T_2 and T_4 generations. Compared to their uncultured parent, Golden Promise, most of these lines were shorter, lower yielding, and had smaller seed, and the variability among individual plants was higher. The frequency and severity of the observed SCV was unexpectedly high, and the transformation procedure appeared to induce greater SCV than tissue culture in the absence of transformation. Attempts to understand the sources of SCV, and to modify transformation procedures to reduce the generation of SCV, should be made.

Key words Barley (*Hordeum vulgare* L.) · Agronomic performance · Somaclonal variation · Transgenic plants · Progeny

Communicated by J. W. Snape

P. Bregitzer (✉)
USDA-ARS, National Small Grains Germplasm Research Facility, P.O. Box 307, Aberdeen, ID 83210, USA

S. E. Halbert[1]
University of Idaho, Aberdeen, ID 83210, USA

P. G. Lemaux
USDA-ARS, Plant Gene Expression Center, Albany, CA, and Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA

Present address:
[1]Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, FL, USA

Introduction

The efficient development of new cultivars is highly dependent on making small, step-wise improvements to existing cultivars or elite breeding lines. Practically, this means that improvements to one or several traits are made without changing the basic genetic background in which these traits are expressed, i.e., no changes are made to the vast majority of traits that define a particular cultivar or breeding line. Improvements may be made by a variety of techniques, but generally breeders simultaneously select for multiple traits within populations derived from similar, genetically elite parents.

Genetic engineering technologies are intended to be adjuncts to this classical, hybridization-based process. To be of the greatest practical value to plant breeders, genetic engineering techniques should produce genetically elite plants with one or more novel characteristics. This goal requires the ability to precisely insert a single gene, or several genes, into existing useful germplasm without inducing mutations.

Virtually all current genetic engineering technologies for barley require that genes be delivered to cells grown in vitro (Jähne et al. 1994; Ritala et al. 1994; Wan and Lemaux, 1994; Hagio et al. 1995; Funatsaki et al. 1995; Yao et al. 1997); however, certain aspects of the in vitro environment are highly mutagenic to cultured tissues (for reviews, see Kaeppler and Phillips 1993; Karp 1994). The in vitro environment can generate spontaneous and heritable genetic changes, termed somaclonal variation (SCV) (Larkin and Scowcroft 1981). Extensive SCV will likely result in many undesirable changes in the genetic background of the transgenic plant. Such plants will probably not function successfully either as cultivars or as genetically elite parents. Introgression of transgenes from such plants into acceptable genetic backgrounds could require multiple cycles of hybridization and progeny analysis. This is particularly true if one or more heritable muta-

tions were closely linked to the transgene or to genes controlling other critical traits, a situation which would limit the value of the genetic engineering approach.

The impact that SCV will have on the application of genetic engineering technologies to barley cultivar development has not yet been documented, but the evidence suggests that SCV may be of considerable importance. Minimal periods of in vitro growth (in some cases as short as four weeks) have resulted in heritable alterations of the methylation patterns of microspore-derived plants (Devaux et al. 1993), alterations in the methylation patterns of plants derived from embryogenic callus and from meristematic cultures (Zhang, Zhang, Bregitzer and Lemaux, unpublished data), and a time-dependent accumulation of cytogenetic aberrations in, and a loss of regenerability of, immature embryo-derived barley callus (Ziauddin and Kasha 1990; Hang and Bregitzer 1993; Bregitzer et al. 1995 a). In addition to changes in nuclear DNA, extensive plastid DNA alterations in albino, microspore-derived barley plants have been documented (Day and Ellis 1985; Dunford and Walden 1991). Subsequent work has provided compelling evidence that such alterations occur during the redifferentiation process (Mouritzen and Holm 1994).

These changes are likely correlated with, if not in part causative of, observed variation in morphological traits, agronomic performance, and malting quality in tissue-culture-derived barley plants. In comparative field performance trials of tissue-culture-derived lines from 18 cultivars or breeding lines of barley, considerable morphological variation was observed (Ullrich et al. 1991). In another replicated field study, protoplast-derived barley plants from the cultivars Igri and Dissa showed significant, negative changes in height, heading date, fertility, spike length, and spikelet density (Kihara et al. 1996). Bregitzer and Poulson (1995) analyzed 30 tissue-culture-derived lines from six barley cultivars in replicated field trials. Agronomic performance, as judged by heading date, height, percentage lodging, grain yield, test weight, and percentage plump kernels, was negatively altered in the majority of the lines. Further analyses of some of these lines also documented reductions in malting quality (Bregitzer et al. 1995 b). Baillie et al. (1992) conducted replicated field trials of 72 tissue-culture-derived lines from four barley cultivars. They noted that some lines had reductions in agronomic performance, although the frequency of such changes was less than that noted by Bregitzer and Poulson (1995).

Many successful methods reported for transformation of cereals involve DNA delivery via microparticle bombardment into cells within scutellar tissues of immature embryos, callus derived from immature embryos, or microspores or microspore-derived embryos, followed by selection during in vitro growth and regeneration of transformed plants. In addition to the apparent mutagenicity of the basic in vitro culturing process, aspects relating to bombardment and selection may induce additional SCV of sufficient severity to pose a serious problem to the utility of transformation procedures. The present study was focused on the agronomic performance of plants derived from transgenic callus of the barley cultivar Golden Promise (Wan and Lemaux 1994) versus seed-derived, non-cultured Golden Promise plants.

Materials and methods

Field trials were conducted in 1994 of 44 T_2 lines representing 15 independent transformation events (families) produced via particle bombardment of immature embryos of the cultivar Golden Promise (GP) (Wan and Lemaux 1994). These trials were grown at Aberdeen and Burley, Idaho, under irrigation, using a randomized complete block design with two replicates. Each plot consisted of a row of ten single plants spaced 14 inches apart. The rows were planted on 28-inch centers to minimize competition between plants. Non-tissue-culture-derived GP, produced in the field, was included as a control. Each T_2 line was produced by bulking seed from 18–30 greenhouse-grown T_1 plants. All families were segregating for the introduced genes *bar* (encoding phosphinothricin resistance) and *uidA* (encoding β-glucuronidase). All families except GP717B-9 were segregating also for a coat-protein gene from barley yellow dwarf virus (pRsa1BYDVcp).

Seven of these lines representing five transformation families in the T_2 and T_4 generations were selected for additional testing in 1996. All plants were selected as null (non-transgenic) segregants based on Basta herbicide susceptibility and negative PCR assays for *bar* and pRsa1BYDVcp. These trials were grown under irrigation at Aberdeen, Idaho, and under dryland conditions at Tetonia, Idaho, using a split-plot design with four replicates. Each plot consisted of 12 single plants for each generation (24 in total), planted in an alternating pattern with multiple-plant-hills of spring wheat such that each barley plant was surrounded by four wheat hills. This provided equal competition for each barley plant. Non-tissue-culture-derived GP, produced in the greenhouse along with the T_2 and T_4 seed, was included as a control. For each line, T_2 and T_4 seed was produced by bulking equal numbers of seeds from each of ten greenhouse-grown T_1 and T_3 plants, respectively. The T_1 seed was from the same source used to produce T_2 seed for the 1994 trials; the T_3 seed was produced in the 1994 trials.

Heading date, height, grain yield, and 100-seed weight data, were collected on an individual plant basis for each transgenic line and the GP control. For each plot, mean values for each trait were calculated based on the individual plant data. A measure of the within-plot variation was obtained by dividing the range of measured values (maximum value–minimum value) within a plot by the mean value for that plot. The means and variability estimates were then subjected to analyses of variance using the GLM procedure within the Statistical Analysis Systems software (SAS Institute 1988). Sources of variation included in the statistical model for 1994 included location, replicates within location, families, lines within families, and appropriate interactions. For 1996, sources of variance were location, replicates within location, lines, generations within line, and appropriate interactions. Dunnett's tests were used to compare transgenic-line performances to control performances.

Results

1994 field trials

Visual observations of the field plots indicated that most of the transgenic plants tested appeared to have

the characteristic morphology of GP, although greater variability was observed among transgenic plants than among GP control plants. Many transgenic lines were less vigorous and matured more slowly than GP (data based on subjective field observations). Morphological variants included plants with a relatively prostrate and spreading growth habit, extreme dwarfism, extremely slow development, several types of chlorophyll mutations, and premature death. The most frequently observed morphological variants are summarized in Table 1.

Within-plot (plant to plant) variability differed significantly ($P = 0.05$) among and within families for height, and among families for grain yield (data not shown). The within-plot variability of the transgenic lines (expressed as a percent of within-plot variability observed for GP) varied from 99 to 221% for height, 131% to 298% for grain yield, and 144% to 534% for 100-seed weight.

Mean agronomic performance differed significantly ($P = 0.05$) among and within families for height, yield, and 100-seed weight. For these traits, 17, 27, and 6 of the 44 transgenic lines showed significant reductions relative to the GP control. Mean family performances for these traits, expressed as percentages of the GP control performance, are summarized in Table 2.

1996 field trials

The morphological variability noted in 1994 was observed in the families tested in 1996. Both the T_2 and the T_4 generations showed similar frequencies of variant plants, although aberrations incompatible with the production of viable seed were less frequent in the T_4 generation. Careful examination of plants of GP717B-14-8 and -12 revealed a low percentage of lateral floret fertility in the majority of the plants. This could not have been caused by outcrossing to a six-rowed barley genotype, because no other morphological traits were segregating in these lines. This abnormality was first noticed in the 1995/96 greenhouse, and likely was present but unnoticed in the 1994 trials.

The significant sources of variation for agronomic performance are shown in Table 3. Examination of the data indicated that the main effects were interpretable despite the significant interactions between location

Table 1 Percentage of individual transgenic plants grown at two locations in 1994 showing the most common morphological variations

Family[a]	# of lines in family[b]	Extreme dwarfism	Semi-prostrate habit	Extremely late maturity
GP717B-2	1	0	12.9	0
GP717B-4	5	1.5	0	0
GP717B-11	2	0.9	0	0
GP717B-14	2	1.5	3.0	1.5
GP717B-31	1	8.6	17.1	1.5
GP717B-32	5	3.7	12.6	3.4
GP717B-33	4	1.6	1.6	0.8
GP717B-59	1	5.3	0	0
GP717B-189	4	2.0	2.0	2.0
GP717B-197	5	1.3	3.1	0
GP724B-1	1	1.6	0	0
GP724B-4	4	3.8	0	0.7
GP724B-47	1	0	0	0
GP724B-96	4	2.7	3.1	0
GP717B-9[c]	4	3.2	0.8	0.8

[a] Each family represents an individual transformation event
[b] Each line derives from an individual regenerated plant
[c] GP717B-9 contains only the selectable marker *bar* and *uidA*

Table 2 Agronomic performances of transgenic barley grown at two locations in 1994, expressed as a percentage of the non-transgenic control

Family[a]	# of lines in family[b]	Height[c]	Yield[c]	100-seed weight[c]
GP717B-2	1	88 (88–88)[d]	56 (56–56)	74 (74–74)
GP717B-4	5	98 (94–103)	85 (69–108)	84 (82–92)
GP717B-11	2	86 (84–88)	54 (50–58)	70 (68–73)
GP717B-14	2	73 (70–76)	16 (16–16)	57 (55–58)
GP717B-31	1	79 (79–79)	47 (47–47)	77 (77–77)
GP717B-32	5	94 (89–97)	66 (53–80)	79 (72–85)
GP717B-33	4	90 (86–93)	64 (57–73)	74 (72–75)
GP717B-59	1	87 (87–87)	64 (64–64)	81 (81–81)
GP717B-189	4	77 (69–87)	27 (16–41)	66 (58–75)
GP717B-197	5	82 (68–96)	49 (21–81)	72 (57–85)
GP724B-1	1	87 (87–87)	45 (45–45)	74 (74–74)
GP724B-4	4	87 (82–90)	60 (42–68)	93 (80–118)
GP724B-47	1	92 (92–92)	79 (79–79)	88 (88–88)
GP724B-96	4	80 (76–88)	50 (40–65)	75 (72–83)
GP717B-9[e]	4	86 (81–92)	50 (43–56)	77 (73–82)

[a] Each family represents an individual transformation event
[b] Each line derives from an individual regenerated plant
[c] Data are expressed as percentages of the non-transgenic GP control performance
[d] Data presented as: family mean (range of line means)
[e] GP717B-9 contains only the selectable marker *bar* and *uidA*

Table 3 Sources of variance for somaclonal variation in transgenic-derived null-segregant barley lines grown in 1996

Source	Trait			
	Heading date[a]	Height	Yield	100-seed weight
Line	–***/NS[b]	***/NS	***/**	***/**
Location × line	---/---	***/NS	***/NS	**/NS
Generation (Line)	NS/NS	**/NS	NS/NS	NS/NS
Loc × gener (Line)	---/---	NS/NS	NS/NS	NS/NS

, * Significant at the 0.01 and 0.001 probability levels, respectively
[a] Heading-date data collected from the Aberdeen location only
[b] Data presented as P value for mean score/P value for estimated deviation.
NS = non-significant

Table 4 Agronomic performances of Golden Promise and transgenic-derived null-segregant barley lines grown in 1996

Genotype	Trait			
	Heading date (Julian)	Height (cm)	Yield per plant (g)	100-seed weight (g)
Golden Promise	193.9	49.6	30.2/1.2[a]	3.7/0.27
GP717B-14-8	196.2*	41.7*	5.2*/2.4*	2.3*/0.61
GP717B-14-12	198.3*	39.6*	4.4*/2.4*	2.3*/0.77*
GP717B-31-3	195.3	47.2*	15.9*/1.8*	3.2*/0.47
GP717B-32-6	195.2	46.3*	15.9*/1.8*	3.0*/0.50
GP717B-32-11	194.3	47.4*	18.2*/1.7*	3.0*/0.43
GP717B-33-3	196.7*	42.7*	12.1*/1.9*	2.8*/0.54
GP724B-4-9	193.6	46.2*	18.0*/1.6	3.4/0.26

* Significantly different from G.P. as determined by Dunnett's multiple comparison procedure ($P = 0.05$)

[a] Data presented as: trait mean/estimated deviation. Estimated deviation = range ÷ trait mean, calculated on a per-plot basis

and line for height, yield, and 100-seed weight. Note also that the effect of generation within line was not significant for any trait, indicating that the agronomic performances of both generations were similar.

Data are therefore presented on a line mean basis in Table 4. Relative to GP, the transgenic-derived null-segregant lines tended to be later-maturing, shorter and lower yielding, and had lower 100-seed weights. The null-segregant lines tended also to have greater variability than GP for yield and 100-seed-weight.

Discussion

The generation of SCV in the transgenic barley lines was expected based on previous observations of non-transgenic barley plants regenerated from callus (Ullrich et al. 1991; Baillie et al. 1992; Bregitzer and Poulson 1995; Bregitzer et al. 1995 b). However, in the current study the frequency and magnitude of the observed SCV was unexpectedly high. For instance, the grain yields of tissue-culture-derived barley lines (R_5 generation) from six cultivars studied by Bregitzer and Poulson (1995) were at least 84% that of their uncultured controls, and the poorest-yielding GP-derived lines in that study averaged 91% of the uncultured GP control. The grain yields of the GP transgenic-derived lines in this study averaged 56% and 42% of the GP control (for the 1994 and 1996 trials, respectively). Although these data came from different studies, the comparison to a common check (Golden Promise), and the magnitude of the differences, suggests that the SCV observed in the current study was greater than that of previous observations. Additionally, the morphological variation observed in this study substantially exceeded previous observations of non-transgenic, tissue-culture-derived plants (Bregitzer, unpublished data). Baillie at al. (1992) detected even less SCV, and concluded that most of their lines did not suffer significant yield reductions as a result of in vitro passage.

In our comparisons of the studies discussed in the preceeding paragraph, we considered several aspects of the protocols used that might have differentially influenced the generation of SCV, such as genotype, explant source, length of culture period, and other specific elements of the in vitro process. Excluding factors specific to the transformation and selection process, the most important potential difference between the protocol of Wan and Lemaux (1994) and the other studies appeared to be the use of dicamba instead of 2,4-D in the production of the transgenic plants. However, we were not convinced that this, or other differences in protocols, could provide a satisfactory explanation for the high levels of SCV observed in the transgenic plants. We therefore considered that the transformation and selection process generated SCV in addition to that caused by in vitro culture per se.

There are a number of potentially mutagenic factors specific to the transformation process that are not part of the basic in vitro process. The use of phosphinothricin as a selection agent results in a locally altered pH and increased ammonia levels, and the transformed cells must grow in a milieu of substances released from dead or dying cells. Consistent with our hypothesis that phosphinothricin-based selection was mutagenic has been our repeated observations that higher proportions of albino plantlets have been regenerated from transgenic tissues subjected to selection than from transgenic tissues not subjected to selection. We have observed this phenomenon in a number of cultivars, including GP (Wan and Lemaux, unpublished 1993; Jiang, Cho, and Lemaux, submitted or unpublished data, 1996). Other potentially mutagenic factors include stresses caused by tissue damage and exposure to vacuum during bombardment.

Increased SCV in transgenic plants versus non-transgenic, tissue-culture-derived plants has been documented in rice (Schuh et al. 1993). In their study, control plants and protoplast-derived non-transgenic plants performed similarly, whereas protoplast-derived transgenic plants had significant reductions in performance (e.g., grain yield approximately 10% of control).

Although the sources of the SCV observed in this study cannot be definitively identified, the performance of the null-segregant lines clearly indicates that the SCV cannot be attributed to effects of transgene insertion or expression. In addition, the similar performances of the T_2 and T_4 generations indicates that the SCV was not caused by non-heritable epigenetic effects.

Regardless of its source, the SCV observed in this study will reduce the utility of these plants as parents in a breeding program. It is tempting to propose backcrossing as a technique to move transgenes from such poor parents into acceptable genetic backgrounds, but this technique could be limited by close linkage of

altered loci to transgenes or to other critical loci. This study did not provide data on such linkages; however, it is reasonable to postulate that a positive correlation exists between the severity of SCV and the frequency of such undesirable linkages. We believe that the long-range goal of transformation should be the non-mutagenic insertion of genes, and SCV is incompatible with that goal.

Further research is needed to understand the exact nature and causes of the observed changes in agronomic performance, and also to understand the practical consequences of these changes. What molecular changes can be identified and correlated to performance in tissue-culture-derived plants? Will the observed SCV in fact be difficult to eliminate via conventional outcrossing to elite germplasm? Does the generation of SCV affect the expression of transgenes? Can changes in the culturing or transformation procedures minimize SCV (such as the elimination of or the use of alternative selective agents, or the use of meristem cultures with minimal dedifferentiation; Ritala et al. 1994; Lowe et al. 1995; Zhong et al. 1996)? Understanding these issues will aid in the utilization of transgenic barley germplasm and in the development of improved, less mutagenic transformation methodologies.

Acknowledgements The authors thank Berry Treat of the Coors Brewing Co. for donating physical facilities used for field trials; Irene Shackelford, Robert Campbell, Amy Miles, Melba Yost, and Rex Anderson for their technical support; and Shibo Zhang, Myeong-Je Cho, David Somers, and Blair Goates for their critical reviews of this manuscript.

References

Baillie AMR, Rossnagel BG, Kartha KK (1992) Field evaluation of barley *(Hordeum vulgare* L.) genotypes derived from tissue culture. Can J Plant Sci 72 : 725–733

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

Bregitzer P, Campbell RD, Wu Y (1995 a) In vitro response of barley (*Hordeum vulgare* L.) callus: effect of auxins on plant regeneration and karyotype. Plant Cell Tiss Org Cult 43 : 229–235

Bregitzer P, Poulson M, Jones BL (1995 b) Malting quality of barley lines derived from tissue culture. Cereal Chem 72 : 433–443

Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr Genet 9 : 671–678

Devaux P, Kilian A, Kleinhofs A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation. Mol Gen Genet 241 : 674–679

Dunford R, Walden RM (1991) Plastid genome structure and plastid-related transcript levels in albino barley plants derived from anther culture. Curr Genet 20 : 339–347

Funatsaki H, Kuroda H, Kihara M, Lazerri PA, Müller E, Lörz H, Kishinami I (1995) Fertile transgenic barley generated by direct DNA transfer to protoplasts. Theor Appl Genet 91 : 707–712

Hagio T, Hirabayashi T, Machii H, Tomotsune H (1995) Production of fertile transgenic barley (*Hordeum vulgare* L.) plants using the hygromycin-resistance marker. Plant Cell Rep 14 : 329–334

Hang A, Bregitzer P (1993) Chromosomal variations in immature embryo-derived calli from six barley cultivars. J Hered 84 : 105–108

Jähne A, Becker D, Brettschneider R, Lörz H (1994) Regeneration of transgenic, microspore-derived, fertile barley. Theor Appl Genet 89 : 525–533

Kaeppler SM, Phillips RL (1993) DNA methylation and tissue-culture-induced variation in plants. In Vitro Cell Dev Biol 29 : 125–130

Karp A (1994) Origins, causes, and uses of variation in plant tissue cultures. In: Vasil IK, Thorpe TA (eds) Plant cell and tissue culture. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 139–151

Kihara M, Takahashi S, Funatsuki H, Ito K (1996) Somaclonal variation in field performance of progeny of protoplast-derived barley plants. Proc Vth Int Oat Conf and VIIth Int Barley Genet Symp, Saskatoon, Saskatchewan, pp 517–519

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60 : 197–214

Lowe, K, Bowen B, Hoerster G, Ross Margit, Bond D, Pierce D, Gordon-Kamm B (1995) Germline transformation of maize following manipulation of chimeric shoot meristems. Biotechnology 13 : 677–682

Mouritzen P, Holm PB (1994) Chloroplast genome breakdown in microspore cultures of barley (*Hordeum vulgare* L.) occurs primarily during regeneration. J Plant Physiol 144 : 586–593

Ritala A, Aspegren K, Kurtén U, Salmenkallio–Marttila M, Mannonen L, Hannus R, Kauppinen V, Teeri TH, Enari T-M (1994) Fertile transgenic barley by particle bombardment of immature embryos. Plant Mol Biol 24 : 317–325

SAS/STAT User's Guide (1988) SAS Institute, Inc, Cary, North Carolina, USA

Schuh W, Nelson MR, Bigelow DM, Orum TV, Orth CE, Lynch PT, Eyles PS, Blackhall NW, Jones J, Cocking EC, Davey MR (1993) The phenotypic characterisation of R_2-generation transgenic rice plants under field conditions. Plant Sci 89 : 69–79

Ullrich SE, Edmiston JM, Kleinhofs A, Kudrna DA, Maatougui MEH (1991) Evaluation of somaclonal variation in barley. Cereal Res Commun 19 : 245–260

Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol 104 : 37–48

Yao QA, Simion E, Williams M, Krochko J, Kasha KJ (1997) Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.). Genome 40 : 570–581

Zhong H, Sun B, Warkentin D, Zhang S, Wu R, Wu T, Sticklen MB (1996) The competence of maize-shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiol 110 : 1097–1107

Ziauddin A, Kasha KJ (1990) Long-term callus cultures of diploid barley (*Hordeum vulgare*). II. Effect of auxins on the chromosomal status of cultures and regeneration of plants. Euphytica 48 : 279–286