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Field performance of transgenic potato plants compared with controls regenerated from tuber discs and shoot cuttings

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Summary. The objective of this study was to separate and determine effects on the field performance of transgenic potatoes that originate from the tissue culture process of transformation and from the genes inserted. The constructs introduced contained the reporter gene for beta-glucuronidase (GUS) under the control of the patatin promoter (four different constructs) and the neomycin phosphotransferase gene under the control of the nopaline synthase promoter. Both genes might be expected to have a neutral effect on plant phenotype. The field performance of transgenic plants (70 independent transformants) was compared with non-transgenic plants regenerated from tuber discs by adventitious shoot formation and from shoot cultures established from tuber nodal cuttings. Plants from all three treatments were grown in a field trial from previously field-grown tubers, and plant performance was measured in terms of plant height at flowering, weight of tubers, number of tubers, weight of large tubers and number of large tubers. There was evidence of somaclonal variation among the transgenic plants; mean values for all characters were significantly lower and variances generally higher than from plants derived from nodal shoot cultures. A similar change in means and variances was observed for the non-transgenic tuber-disc regenerants when compared with shoot culture plants. Plant height, tuber weight and tuber number were, however, significantly lower in transgenic plants than in tuber-disc regenerants, suggesting an effect on plant performance either of the tissue culture process used for transformation or of the genes inserted. There were significant differences between constructs for all five plant characters. The construct with the smallest segment of patatin promoter and the lowest level of tuber specificity for GUS expression had the lowest values for all five characters. It is proposed that the nature of GUS expression is influencing plant performance. There was no indication that the NPTII gene, used widely in plant transformation, has any substantial effect on plant performance in the field.

Key words: *Solanum tuberosum* – Transformation – Somaclonal variation – Beta-glucuronidase – Neomycin phosphotransferase

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Introduction

Various genes have been introduced by transformation into potato and other crop plants (see for example Lycett and Grierson 1990). The evaluation of transgenic plants is first carried out under containment conditions in culture rooms and glasshouses and this gives valuable information on expression of the introduced gene. However, an overall assessment of plant phenotype and performance can only be made by testing transgenic plants in a field environment, with its inherent environmental variations. To-date field evaluations of transgenic plants have been principally used to study the efficiency of introduced genes for specific functions such as disease resistance (Nelson et al. 1988), pest resistance (Delannay et al. 1989) and herbicide resistance (De Greef et al. 1989; D'Halluin et al. 1990). There are no reports of studies assessing quantitative plant performance characters in a range of independently transformed plants under standard field conditions. More specifically, there is only one field study (McHughen and Holm 1991) which examines whether there are effects on plant phenotype, in addition to those expected from the introduced gene, that are associated with the process of transformation or the transgenic state.

The performance of transgenic plants can be affected by three phenomena. (1) *Insertion mutagenesis*. DNA introduced by transformation can cause disruption of the plant gene it inserts into or close to (Feldmann and Marks 1987; Feldmann et al. 1989). Disruption of major genes to give an obvious change in a plant character is most visible, but it is likely that insertion mutagenesis also causes more subtle changes in phenotype. (2) *Pleiotropy*. It is known from genetical studies with non-transgenic plants that individual genes can have, apparently unrelated, multiple effects on plant phenotype. (3) *Somaclonal variation*. The creation of transgenic plants usually involves a tissue-culture phase and the regeneration of plants from explants with a high capacity for plant regeneration. Plant regeneration from cultured tissues and cells is well known to result in genetic variation among regenerated plants (Larkin and Scowcroft 1981; Karp and Bright 1985; Karp 1991).

The aim of the experiment reported here was to compare, under agricultural field conditions, potato plants derived from three sources: transgenic plants derived from co-cultivation of tuber discs with *Agrobacterium tumefaciens*; plants regenerated from tuber discs without transformation, and plants established from tuber nodal shoot cuttings. Plants from shoot cuttings do not pass through a disorganised tissue culture state and are often used to maintain genetically stable lines (Westcott et al. 1977; Dale et al. 1986). Plants regenerated from tuber discs without transformation should give an estimate of somaclonal variation (Dale et al. 1986; Rietveld et al. 1991) and those with transformation, an estimate of effects associated with the transformation process or state.

Materials and methods

The transgenic and non-transgenic plant material

Solanum tuberosum L. variety Desiree was transformed using co-cultivation of tuber discs with *A. tumefaciens* (Sheerman and Bevan 1988). The transgenic plants were provided by M. Bevan and R. Jefferson and contained constructs 141.1, 141.2, 141.3 and 141.4 described elsewhere (Jefferson 1990; Jefferson et al. 1990). These consisted of class 1 patatin promoter fragments 369, 674, 957 and 2,164 bp from the transcription start site, respectively. Transcriptional fusions were made with the gene encoding GUS (beta-glucuronidase, Jefferson 1989) and terminated by the polyadenylation sequences of the nopaline synthase gene. The four constructs were introduced into the pBin19 binary vector plasmid (Bevan 1984) containing the NPTII (neomycin phosphotransferase) gene for resistance to kanamycin, with the nopaline synthase promoter and terminator. The 70 transgenic plants came from different transformation events.

Non-transgenic control plants were established from tuber nodal cuttings and from adventitious shoots regenerated from tuber discs. To establish nodal cuttings, shoots were taken from sprouting tubers, surface sterilized in 10% sodium hypochlorite for 10 min, washed six times in sterile water and transferred to 5 cm plastic Petri dishes containing MS basal medium (Murashige and Skoog 1962) with 7 g/l agar (Sigma A1296). Plants were regenerated from tuber discs, using the same protocol as for transformation (Sheerman and Bevan 1988), in the presence of an auxin (IAA aspartic acid) and a cytokinin (zeatin riboside) without *A. tumefaciens* co-cultivation and kanamycin selection.

Production of tubers for planting

The three classes of regenerated plants, all established from healthy tubers, (Table 1) were propagated by nodal cuttings taken monthly to give sufficient in vitro plants for transfer to the glasshouse and eventually to the field plot. Samples of in vitro plants were tested using the ELISA method (Clark and Adams 1977) for X, Y, and Leaf Roll viruses and found to be negative. In vitro plants were first transferred to peat pots (5 cm diameter) in a glasshouse and after 4–6 weeks transferred into a randomized and replicated field plot and irrigated to improve establishment. To avoid any complications of physiological variability in plant and tuber characteristics, arising from establishing the field experiment from in vitro-derived plants, tubers were taken from this first year and planted in a second year. All the data presented is, therefore, from a replicated and randomized experiment in the tuber-planted material of the second year (Table 1).

Testing

The field trial plot was prepared in the same way as for the standard potato breeding trials at the former Plant Breeding Institute in Cambridge. These operations included fertilizer application, cultivations, and spraying against blight and insect pests. Planting was at 46 cm tuber spacing (approximately twice the normal plant spacing) along ridges 76 cm apart. The growing plants were inspected for disease throughout the season and mild symptoms of Leaf Roll virus were observed only rarely in plants distributed at random throughout the plot.

At maturity various plant characters were scored, with particular emphasis on tuber production. The height of the tallest stem was measured from the soil surface to the stem apex. Lax stems were straigthened by lifting to obtain their linear height. After tuber harvest (see below) the following characters were determined for each plant: total weight of tubers, total number

Table 1. Number of regenerated plants (variety Desiree) used for field assessment

Sources of plants	Number of independently transformed plants or regenerants [a]
Tuber node shoot cultures [b]	41
Tuber disc adventitious shoot regenerants [b]	45
Transformed adventitious shoots from tuber discs	
Construct 1 (141.1) [c]	30
Construct 2 (141.2)	14
Construct 3 (141.3)	10
Construct 4 (141.4)	16

[a] Each of the 156 lines was represented by one plant in each of three replicates

[b] Each source taken from a range of tubers

[c] Numbers in brackets correspond to the construct numbers used by Jefferson et al. (1990)

of tubers, weight of large tubers not able to pass through a 50 cm sieve and number of the same large tubers.

The field trial was carried out according to guidelines of the UK Advisory Committee on Genetic Manipulation [ACGM; covered by MAFF licence No. 48A/114(57)] and appropriate procedures followed.

Results

Where the three tubers selected for planting from each of the 156 lines (Table 1) were of different sizes, the largest tuber was planted in replicate 1, the intermediate in replicate 2 and the smallest in replicate 3. Using an analysis of variance, significant differences for the characters measured were generally observed between replicates (data not shown). Replicate 1 frequently had significantly higher values for tuber yield than replicate 3, with replicate 2 intermediate.

One of the transgenic plants had white tubers instead of the normal red, but otherwise there were no obvious and easily visible phenotypic differences between the three sources of plants.

Comparisons of transgenic plants with those from shoot cultures

For all five characters the means were significantly lower for the transgenic plants than for the shoot-culture derived plants (Table 2). Plants from both sources were handled in the same way through in-vitro propagation, establishment in the glasshouse, and growth in the field in the first year, to give tubers for planting in this experiment. Therefore, differences between the transgenic plants and the shoot-culture regenerants are likely to result from the introduced DNA or from the tissue culture step associated with the transformation process.

Somatic of somaclonal variation

It is known that somaclonal variation is observed for yield characters among potato plants regenerated from tuber discs using the same (Dale et al. 1986) or similar (Rietveld et al. 1991) plant regeneration protocols as used here but without the transformation step. Because of this, plants regenerated from tuber discs were also included in the present study to give an estimate of somaclonal effects in the absence of transformation.

For four out of the five characters (Table 2) the mean values were significantly lower for plants regenerated from tuber discs than from plants derived from shoot cultures. However, the mean values for transgenic plants were also significantly lower than the tuber-disc regenerants for three out of the five characters. This suggests that there is somaclonal variation among the tuber-disc regenerants and between the transgenic plants, but because the means for two characters are significantly lower in the transgenic plants compared with the tuber-disc regenerants, there may also be an effect of the introduced genes on plant performance. Because of the difficulty of genetic analysis in potato, the genetic basis of the somaclonal variation could not be determined.

Comparison of variances

Somaclonal variation, like other sources of mutation, is essentially a random process which would be expected to increase variation among affected plants. The distributions of values for three of the plant characters are illustrated in Fig. 1 and it is clear that the distribution is different for the transgenic plants, the tuber-disc regenerants and the shoot-culture plants. A comparison of variances (Table 3) shows that there is heterogeneity between them, and that there is generally more variation between plants regenerated from tuber discs and transgenic plants than between plants from shoot cultures. There is also an indication (for four out of the five characters) of there being more variation between transgenic plants then between the tuber-disc regenerants.

Comparison between transgenic plants transformed with the four constructs

More insight into the effects of transformation on the performance of the transgenic plants can be obtained

Table 2. Mean performance for plant and tuber characters between plants derived from in-vitro shoot cultures, tuber-disc regenerants and transformed shoots from tuber discs

Plant origin	Number of lines	Mean plant height (cm)	Mean weight of tubers per plant (gm)	Mean number of tubers per plant	Mean weight of large tubers per plant (gm)[a]	Mean number of large tubers per plant
Shoot cultures	41	88.8 A[b]	1,297 A	10.4 A	1,034 A	5.1 A
Tuber-disc regenerant	45	85.6 B	1,096 B	9.9 A	726 B	3.6 B
Transgenic cultures	70	83.7 C	941 C	8.2 B	654 B	3.3 B

[a] Tubers too large to pass through a 50 mm sieve

[b] Homogeneity class, LSD $P = < 0.05$. For each character, values with the same letters are not significantly different at a probability of < 0.05. Statistical analysis was by analysis of variance using the Protected Least Significant Difference method (Snedecor and Cochran 1980)

Table 3. The between plant variances for five plant and tuber characteristics from plants derived from in-vitro shoot cultures, tuber-disc regenerants and transformed shoots from tuber discs

Plant origin	Plant height[a]	Weight of tubers	Mean number of tubers	Mean weight of large tubers	Mean number of large tubers
Shoot cultures	28.21 (66–98)	32,598 (896–1,658)	1.85 (7.2–13.3)	34,097 (653–1,441)	0.78 (3.6–7.1)
Tuber-disc regenerants	26.64 (69–104)	40,410 (512–1,457)	4.35[b] (5.6–17.7)	67,654 (17–1,191)	1.35 (0.1–5.7)
Transgenic plants	63.74 (59–97)	60,991 (275–1,477)	2.88 (3.8–12.5)	71,680 (0–1,212)	1.6 (0–5.7)
Bartlett's test probability value	<0.01	NS (<0.1)	<0.05	<0.05	<0.05

[a] Units of measurement are given in Table 2. Range of mean plant values are given in brackets. Variances are compared using Bartlett's homogeneity of variances test (Snedecor and Cochran 1980)
[b] Excluding a single high mean plant value of 17.7 tubers from a plant with almost entirely small tubers, this variance value is 2.95 with a range of 5.6–13.9

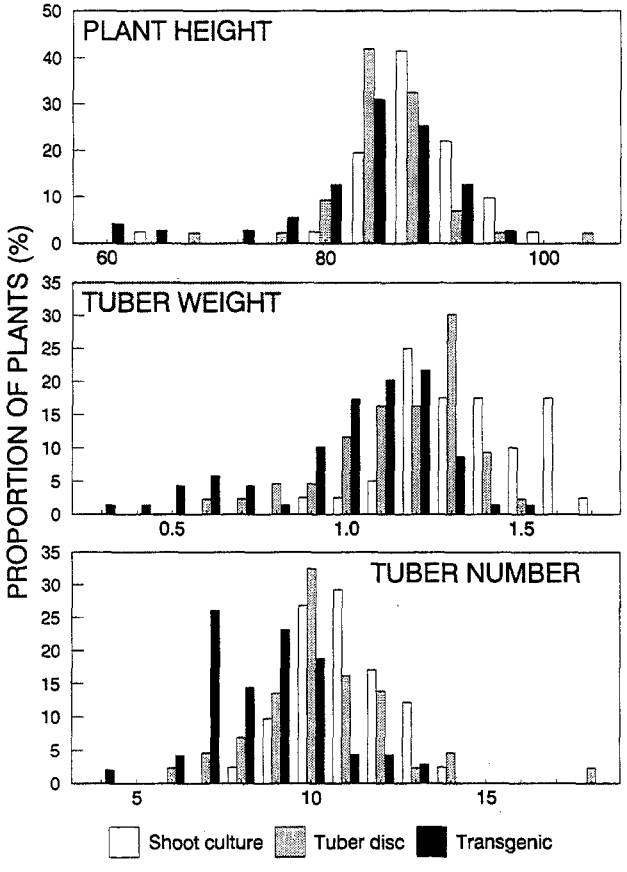

Fig. 1. Histograms showing the distribution of plants for height (cm), mean total tuber weight per plant (kg), and mean total tuber number per plant, from plants derived from shoot cultures, tuber-disc regenerants and transgenic regenerants

from a comparison of plants transformed with different constructs. The four constructs contained different fragments of the patatin promoter regulating the GUS gene. In other respects the constructs were the same. There are highly significant differences overall ($P<0.01$) between constructs for all five characters measured (Table 4). When individual constructs are compared there are no significant differences between constructs 2, 3 and 4 for four out of the five characters. Plants containing construct 1 have the lowest values recorded for all characters and the differences are statistically significant from the other constructs in many cases. For example, the performance of plants containing construct 1 are on average significantly lower than those containing construct 2 in four out of the five characters.

In conclusion there is a marked difference in plant growth between transgenic plants containing different constructs and suggests that the GUS gene regulated by the patatin promoter is having an effect on plant performance.

The effect of the kanamycin resistance gene

All constructs contained the NPTII gene, so it is not possible directly to compare the plant performance of transgenics with and without this gene. However, the data does contain some information on the effect of the NPTII gene on plant performance. Compared with the controls, in general, construct 1 has the greatets effect on plant performance and construct 2 the least. The most appropriate control plants in this case are those from tuber discs because they, like the transgenic lines, contain any somaclonal effects. Any difference in plant performance between plants with construct 2 and those from tuber discs will contain effects of the GUS gene in that construct and the NPTII gene. If the differences in performance between construct 2 plants and the tuber disc

Table 4. Mean performance for plant and tuber characteristics between transgenic plants containing four different DNA constructs

Construct	Number of transgenic lines[a]	Mean plant height (cm)	Mean weight of tubers per plant (gm)	Mean number of tubers per plant	Mean weight of large tubers per plant (gm)	Mean number of large tubers per plant
1	30	81.3 A[b]	849 A	7.6 A	566 A	2.8 A
2	14	87.0 B	1,031 B	8.2 AB	794 B	3.9 C
3	10	84.6 AB	1,003 B	9.3 B	650 AB	3.2 AB
4	16	84.1 AB	997 B	8.7 B	690 B	3.5 BC

[a] Each line is from an independent transformation event and had three individual plants among three replicates
[b] Homogeneity class, LSD $P<0.05$ (see Table 1)

control are small, we can conclude that the effects of the NPTII gene are also likely to be small. Data from the tuber-disc regenerants and from plants containing construct 2 (with significance levels) are respectively as follows: mean plant height 85.6 and 87.0 ($P=0.28$), mean weight of tubers per plant 1,096 and 1,031 g ($P=0.16$), mean number of tubers per plant 9.9 and 8.2 ($P=<0.01$), mean weight of large tubers 726 and 794 ($P=0.23$), and mean number of large tubers 3.6 and 3.9 ($P=0.20$). There is a significant difference for one out of the five characters.

Discussion

Presence of somaclonal variation

There is good evidence for the presence of somaclonal effects among plants regenerated from tuber discs, from the significant shift in mean values of the characters and the generally higher variances when compared with shoot-culture-derived plants. Rietveld et al. (1991) also observed an increase in variance for 13 of the 22 characters they measured in potatoes regenerated from tuber discs. This was frequently accompanied by a significant shift in mean value, but not always in the downward direction as observed in this study. The differences in means and variances between transgenic plants and shoot culture plants, as would be expected, also provides evidence of somaclonal variation among the transgenic plants.

The observation of lower mean values for transgenic plants compared with tuber-disc regenerants and the trend towards higher between-plant variances, suggests additional effects to those of somaclonal variation. These may be effects attributable to insertion mutagenesis or to an influence of the introduced genes. However, it should be noted that transformation and the selection of transformed-cells imposes intense selection on a minority of cells that integrate functional copies of the T-DNA from *Agrobacterium*. The process of selection and the proliferation of cells from within tissues in which the majority of cells are inhibited from dividing by the antibiotic may produce a different pattern and degree of somaclonal variation compared with plants regenerated from tuber discs without these developmental constraints.

The possibility of T-DNA insertion mutations

There are two reasons why T-DNA insertional mutagenesis is unlikely to be having any significant influence on the performance of the transgenic plants in the experiment reported here. The *first* is that the majority of mutations are known to be recessive (Fincham 1983) and, therefore, would not be visible in the transformed generation (T_0). It would be necessary to proceed to later generations of self pollination, to the T_2 in tetraploid potato for genes in the quadriplex condition (four dominant alleles at the same locus; Allard 1960), to observe the phenotypic expression of a recessive mutation. The *second* reason is that in most higher plants, including potato, there is a high proportion of the DNA that is not actively coding (Feldmann et al. 1989). Although there is evidence now that the position of T-DNA insertion may not be at random within the plant genome (Errampalli et al. 1991), any T-DNA copies integrating into inactive DNA would not be expected to have any effect on plant phenotype.

One of the transgenic plants had white tubers whereas the variety Desiree has red-skinned tubers. The origin of this was probably somaclonal variation, as white tuber types are common among somaclones (Wheeler et al. 1985).

Pleiotropic effects of introduced genes

In most studies of this kind, genes are introduced with the intention of modifying phenotype. In this experiment, the NPTII gene and the GUS gene would not be expected to have any overt influence on the characters measured. A comparison between transgenic plants, however, revealed significant differences between constructs. The NPTII gene was common to all constructs so any difference between constructs will not be a direct result of this antibiotic resistance gene. The primary difference between constructs is in the size of the patatin

promoter and construct 1 has the smallest promoter fragment. The reason for the poorer field performance from construct 1 transgenic plants compared with those containing a larger patatin promoter fragment may be associated with the mode of action of the promoter which controls expression of the patatin glycoprotein in tubers. Analysis of the effects of these promoters by Jefferson et al. (1990), showed that construct 1 gave reduced tuber specificity compared with the other constructs, with significant levels of GUS expression in stems and root tissues. For example, the ratio of GUS expression in leaves: tubers was 1:100 for construct 1 compared with 1:2,000 for construct 4. It is possible, therefore, that the expression of GUS in shoot tissue of plants with construct 1 has a more disruptive effect on plant growth than in plants containing the other constructs which expressed GUS more specifically in tubers. This phenomenon could, clearly, be defined as a transgene having a pleiotropic effect on plant phenotype. The precise mechanism by which construct 1 is modifying growth requires further study, but the findings confirm the importance of the analysis of regulatory sequences and studies on the ways in which variation in spatial and temporal expression of a particular transgene affect plant phenotype.

Conclusions

It is important to determine whether there are any unanticipated and subtle effects from genes introduced by transformation or from somaclonal variation, and the work reported here emphasises the need for early field evaluation of transgenic plants for agronomic and risk-assessment purposes. In practice, several transgenic plants need to be obtained from a construct so that genotypes with unwanted somaclonal variation can be discarded and plants with acceptable expression of the introduced genes selected. Levels of expression between different transgenic plants can vary considerably (Hobbs et al. 1990; Blundy et al. 1991). The distribution of agronomic character-values for transgenic plants (Fig. 1) shows that many lie within the distribution of the shoot-culture controls, so it should be possible to select plants which have both desirable levels of transgene expression and no or minimal somaclonal variation.

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