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Facilitating the recovery of phenotypically normal transgenic lines in clonal crops: a new strategy illustrated in potato

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Abstract Transgenic plants frequently exhibit altered phenotypes, unrelated to transgene expression, which are attributed to tissue culture-induced variation and/or insertional mutagenesis. Distinguishing between these possibilities has been difficult in clonal crops such as potato, due to their highly heterozygous background and the resulting inherent phenotypic variability associated with segregation. This study reports the use of transgene integration as a molecular marker to trace the clonal origin of single cells in tissue culture. Following transformation, multiple shoots have been regenerated from cell colonies of potato (*Solanum tuberosum* L.) and Southern analysis used to confirm their derivation from a single transformed cell. Analysis of phenotypic variation in field trials has demonstrated marked differences between these multiple regeneration events, the origin of which must have occurred after T-DNA insertion, and consequently during the tissue culture phase. This result unequivocally demonstrates that somaclonal variation occurs during tissue culture and independent of transgene insertion. Furthermore, the first shoots recovered do not necessarily exhibit less somaclonal variation, since later regeneration events can give rise to plants that are more phenotypically normal. Therefore, when developing transgenic lines for genetic improvement of clonal crops, multiple shoots should be regenerated and evaluated from each transformation event to facilitate the recovery of phenotypically normal transgenic lines.

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Introduction

Somaclonal variation refers to the commonly observed phenomenon of variant plants following regeneration from somatic tissue during in vitro culture (Larkin and Scowcroft 1981), and has been reported for all species studied (Phillips et al. 1994, Veilleux and Johnson 1998). The variation may have either a heritable or epigenetic basis, and may effect changes in the plant through a variety of mechanisms, e.g. chromosomal aberrations including ploidy changes, activation of transposable elements, DNA methylation changes, or point mutations (Veilleux and Johnson 1998). Once considered a valuable new tool for breeding new characters into plants, somaclonal variation is now considered to be something to be avoided in applications such as the production of transgenic plants (Karp 1991; Conner and Christey 1994; Phillips et al. 1994; Conner 2007).

Transgenic plants frequently exhibit altered phenotypes unrelated to the expression of the transferred genes. These changes are usually attributed to somaclonal variation and/or insertional mutagenesis, but it has been difficult to conclusively differentiate between these two options (Veilleux and Johnson 1998). Furthermore, somaclonal variation is generally considered to arise from both pre-existing somatic variation in the source tissue of the parent plant and variation induced during the tissue culture phase (Veilleux and Johnson 1998; Duncan 1997). Distinguishing between these options is highly problematic and is largely based on circumstantial evidence associated with observations that regenerated plants exhibit higher frequencies of phenotypic variation upon longer periods in culture and observed variation between plants assumed to have been derived from the same single cell in culture. However, such evidence remains to be equivocal in the absence of a molecular

marker to trace the origin of single somatic cells after they have been established in culture.

Southern analysis of DNA fragments that encompass the integration site of transgenes in plant genomes provides a unique identifier for each specific transformation event (Deroles and Gardner 1988). In this paper we use *Agrobacterium*-mediated T-DNA transfer to tag single potato (*Solanum tuberosum* L.) cells with a unique molecular marker as they are placed into tissue culture, and evaluate in field trials the variation in morphological traits among multiple regeneration events derived from the same transformed cell. The findings have important implications for the development of transgenic cultivars for commercial release in clonal crops.

Materials and methods

Potato transformation

Virus-free potato plants of cultivars Iwa, Red Rascal, Karaka and Pacific were multiplied in vitro on MS salts and vitamins (Murashige and Skoog 1962), plus 30 g/L sucrose, 40 mg/L ascorbic acid, 500 mg/L casein hydrolysate and 7 g/L agar, adjusted to pH 5.8 with 0.1 M KOH. Plants were routinely subcultured as 2–3 node segments every 3–4 weeks and incubated at 26°C under cool white fluorescent lamps (80–100 μmol/m^2/s; 16 h photoperiod). Leaves were excised from the in vitro plants and transformed as previously described (Barrell et al. 2002) using the disarmed *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) harbouring either of two binary vectors: pART27G14 with a chimeric *cry*9Aa2 gene (Gleave et al. 1998) or pBINMgC [a derivative of pBINPLUS (van Engelen et al. 1995) with an inserted chimeric magainin gene (Barrell and Conner 2009)]. In brief, the leaves were cut in half and dipped for about 30 s in the liquid *Agrobacterium* culture, then blotted dry on sterile filter paper. The leaf segments were cultured on callus-induction medium [potato multiplication medium defined above, supplemented with 0.2 mg/L NAA and 2.0 mg/L BAP], and incubated under highly reduced light intensity (5–10 μmol/m^2/s) by covering the culture vessels with white paper. Two days later, the leaf segments were transferred to the callus-induction medium supplemented with 200 mg/L Timentin to prevent *Agrobacterium* overgrowth. After a further 5 days, leaf segments were transferred onto the same medium further supplemented with 100 mg/L kanamycin. Small cell colonies (0.5–1 mm diameter) developing within 2–6 weeks were picked from the leaf surface and transferred to a shoot regeneration medium (potato multiplication medium with sucrose reduced to 5 g/L, plus 5 mg/L GA_3, 1.0 mg/L zeatin, 200 mg/L Timentin, and 100 mg/L kanamycin). These were cultured under low light intensity (30–40 μmol/m^2/s) until shoots regenerated. Independently derived shoots regenerating from each colony were excised and transferred to potato multiplication medium supplemented with 100 mg/L Timentin. Once complete potato plants had developed, they were subcultured and rechallenged with the 50 mg/L kanamycin under a higher light intensity (80–100 μmol/m^2/s). All media were sterilised by autoclaving at 103 kPA for 15 min. Filter sterilised antibiotics were added, as required, just prior to dispensing media into culture vessels.

Southern analysis

Genomic DNA was isolated from in vitro shoots of the kanamycin-resistant potato lines using Plant DNAzol (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. A total of 10 μg of DNA per line was digested with *Eco*R1, restricting once within the T-DNA of the appropriate binary vector at either side of the *cry*9Aa2 or magainin transgene. This DNA was used for Southern analysis as described by Sambrook et al. (1989) using Hybond N^+ (Amersham, Uppsala, Sweden) membrane and probed according to manufacturer's instructions. The probe used in the Southern analysis consisted of a fragment corresponding to the coding region of the respective transgenes.

Field trial assessment

Each transgenic line was clonally multiplied via micropropagation, transferred to a containment greenhouse and hardened-off using our standard procedures (Conner et al. 1994). The resulting plants were used for establishing the field trial. Rows of the potato plants were spaced 75 cm apart, with 30 cm between plants within rows. Ten plants of each line were transplanted as a single plot within a row, with a 1 m gap between plots. Plots of these transgenic lines were interspersed with plots of the appropriate non-transgenic controls. The experimental plots were completely surrounded by plots from trials on other transgenic potatoes or three buffer rows of non-transgenic potato to prevent "edge effects" during the trial. Weeding was done by hand and potato plants were subjected to overhead irrigation as required during crop establishment. Rows were mounded once, 4 weeks after planting. The experiment was repeated in a contrasting environment by growing plants under simulated field conditions in an insect-proof screenhouse covered with 30-gauge mesh gauze. Sprouted tubers were individually planted into black polyethylene 'PB28 Planta bags' (26 cm diameter, 30 cm high) using our standard soil-mix (Conner et al. 1994). Ten replicate plants of each line and the non-transgenic control were grown in completely randomised design and watered as required. Throughout all trials observations were made on the general

appearance of the transgenic lines and physical measurements of vegetative traits were made 15 weeks after planting on the youngest fully expanded leaf on each plant. At harvest, the number of tubers and weight of tubers per plant were recorded. Data from measurements of morphological traits were subjected to a standard Model I analysis of variance with equal sample sizes (Sokal and Rolf 1969).

Flow cytometry

Genome size in the transgenic lines was determined by flow cytometry as described by Morgan et al. (1995). In brief, nuclei were isolated following the chopping of leaves in Galbraith's buffer, treated with RNAase (DNAase-free) and stained with 50 mg/mL propidium iodide. They were then analysed on an Epics Profile II flow cytometer (Beckman Coulter, Fullerton, CA, USA), fitted with an argon laser (488 nm) that operated at 15 mW. *Hordeum vulgare* L. (barley, cultivar Sultan, 11.12 pg DNA/2C nucleus) was used as an internal standard with each sample.

Results

Several series of multiple shoots were regenerated from individual potato cell colonies derived from cultivars Iwa, Red Rascal, Karaka and Pacific transformed with pART27G14, as well as cultivar Iwa transformed with pBINMgC. The number of independently derived lines regenerated from each cell colony varied from 2 to 10 per cell colony, the majority of which were confirmed by Southern analysis as originating from the same transformed potato cell. Each series of lines was established in a field trial. For simplicity, representative data from only one series of transgenic lines derived from each vector are presented.

Initial data analysis involving the replicated plots of non-transgenic control plants found no evidence of any field trends. (Consequently comparisons among the plots of different regeneration events derived from the same transformed cell provide a true test for phenotypic variation occurring in tissue culture). In all instances the ten clonally micropropagated plants derived from each shoot exhibited a highly consistent phenotype within each line in the field. However, among the different regeneration events within each series, different phenotypic appearances were observed between some lines. For example, in a series of five transgenic lines (DG4a-e) derived from the same cell transformed with pART27G14, the first and second regenerated lines (DG4a and DG4b) were highly uniform within each line, yet markedly different from each other (Fig. 1). All five lines in the DG4 series had the same DNA content (3.8 pg/2C nucleus), equivalent to the original potato

Fig. 1 Transgenic potato lines from the DG4 series, representing potato cultivar Iwa transformed with pART27G14 containing a chimeric *cry*9Aa2 gene. **a** Five lanes of a Southern blot confirming that the DG4 series arose from the same transformed cell. DNA was cut with *Eco*RI, which cuts once within the T-DNA on the right border side of the *cry*9Aa2 gene, and the blot probed with the coding region of the *cry*9Aa2 gene. All regeneration events have three T-DNA integration sites with flanking left border fragments of identical size [12 (*arrow*), 10 and 9 kb]. **b** Field plots of DG4b (*left*) and DG4a (*right*) from the DG4 series each with ten uniform micropropagated plants, but exhibiting markedly different levels of phenotypic variation

cultivar, yet they exhibited different tuber yield parameters (Table 1).

Another series included ten independently derived transgenic lines (MgC20) regenerated from the same cell transformed with pBINMgC. Southern analysis established that all ten plant lines had the same hybridisation patterns at both the left and right border fragments when probed with the inserted transgene, proof that they all arose from the same transformed cell. For example, upon digestion with

Table 1 Genome size and yield performance (mean ± SE) in the field on the DG4 series of transgenic lines derived from the same original transformed cell, plus the non-transgenic control of the parental cultivar Iwa (n = 10)

Line	DNA content (pg/2C nucleus)	Number of tubers/plant	Total tuber weight/plant (g)	Weight/tuber (g)
DG4a	3.8	4 ± 1	57 ± 5	17 ± 2
DG4b	3.8	7 ± 1	425 ± 70	62 ± 5
DG4c	3.8	11 ± 2	674 ± 96	63 ± 6
DG4d	3.8	15 ± 1	888 ± 77	61 ± 3
DG4e	3.8	4 ± 1	80 ± 9	20 ± 3
Iwa control	3.8	8 ± 1	891 ± 52	108 ± 6
[a]F_s [d.f. = 5,59]	–	28.24***	47.89***	70.62***
LSD (5%)	–	2	153	11

[a] F_s value from analysis of variance

*** Represents significant differences between means at the 0.1% probability level

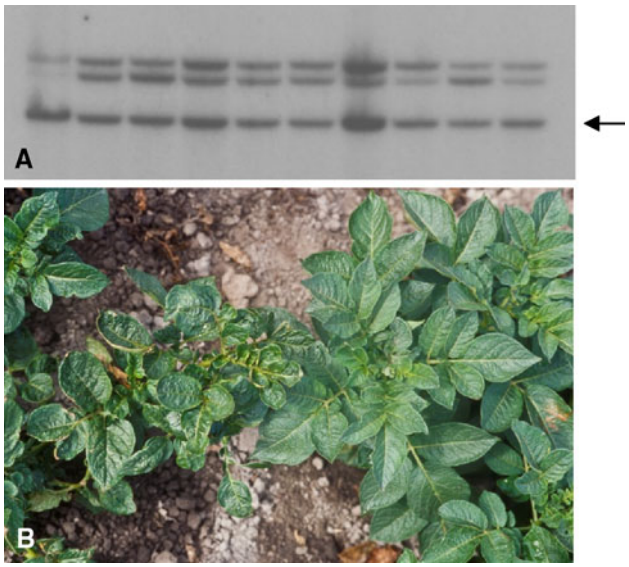

Fig. 2 Transgenic potato lines from the MgC20 series, representing potato cultivar Iwa transformed with pBINMgC containing a chimeric magainin gene. **a** Southern analysis confirming that all ten lines arose from the same transformed cell. DNA was cut with *Eco*RI, which cuts once within the T-DNA on the right border side of the inserted transgene, and the blot probed with the coding region of the magainin gene. In all ten regeneration events, three T-DNA integration sites with flanking left border fragments of identical size [approximately 4.5 (*arrow*), 5.5 and 6 kb] were evident, confirming that all ten lines originate from the same single transformed cell. **b** The differing phenotypic appearance of foliage in field plots of MgC20e (*left*) and MgC20f (*right*) from the MgC20 series

*Eco*RI, three identical left border T-DNA flanking fragments of approximately 4.5, 5.5 and 6 kb were present in all ten lines (Fig. 2a). Despite being derived from the same transformed cell, these ten lines varied markedly in morphological appearance involving foliage traits and tuber yield (Table 2). In the MgC20 series, this was especially evident in line MgC20e (from the fifth shoot to regenerate) which exhibited smaller, curled and rounded leaflets compared with line MgC20f (from the sixth shoot to regenerate), which displays a more normal phenotype (Fig. 2b).

The experiment on the DG4 and MgC20 series of potato lines was repeated in a different environment by growing plants under simulated field conditions in an insect-proof screen-house. The results obtained were consistent with the previous assessment and confirmed the highly consistent phenotypic appearance of plants within the different regeneration events, but the markedly different performance between independent regeneration events from the same transformed cell (Tables 3, 4).

Discussion

When developing transgenic lines for genetic improvement of clonal crops, the aim is to recover a transgenic line with the desired transgene expression while retaining all the elite genetic attributes of the parental clone (Conner and Christey 1994). The initial transformed line selected in tissue culture is therefore propagated as a vegetative clone through to commercial release. This asexual reproduction immediately fixes the initial hemizygous status of the transgene within the highly heterozygous genetic background of clonal cultivars. One of the major limitations to identifying appropriate transgenic lines is the occurrence of phenotypic 'off-types' among populations of transgenic plants.

Somaclonal variation among plants regenerated from tissue culture is well known in many species (Phillips et al. 1994; Veilleux and Johnson 1998), and has been frequently observed under field conditions in potato (e.g. Shepard et al. 1980; Secor and Shepard 1981; Potter and Jones 1991; Thieme and Griess 2005). Although rare somaclonal events can represent desirable attributes, the observed changes usually have negative impacts on plant performance.

Table 2 Genome size and mean values ± SE for eight quantitative traits from field measurements on the MgC20 series of transgenic lines derived from the same original transformed cell, plus two independent non-transgenic controls of the parental cultivar Iwa (n = 10)

Line	DNA content (pg/2C nucleus)	Number of shoots/plant	Plant height (cm)	Leaf length (cm)	Terminal leaflet length (mm)	Terminal leaflet width (mm)	Number of tubers/plant	Total tuber weight/plant (g)	Weight/ tuber (g)
MgC20a	3.7	6 ± 0.4	49 ± 2	13 ± 0.4	73 ± 2	43 ± 1	8 ± 1	736 ± 69	89 ± 5
MgC20b	3.7	8 ± 0.5	47 ± 1	11 ± 0.2	63 ± 2	36 ± 1	11 ± 1	684 ± 31	64 ± 7
MgC20c	3.7	6 ± 0.5	44 ± 1	11 ± 0.5	66 ± 2	36 ± 2	8 ± 1	628 ± 57	74 ± 5
MgC20d	3.7	6 ± 0.2	50 ± 2	12 ± 0.3	67 ± 2	35 ± 1	9 ± 1	719 ± 35	78 ± 3
MgC20e	3.7	4 ± 0.3	42 ± 3	9 ± 0.4	56 ± 3	30 ± 1	6 ± 1	325 ± 47	53 ± 5
MgC20f	3.7	7 ± 0.6	39 ± 2	10 ± 0.4	60 ± 2	34 ± 2	8 ± 1	623 ± 60	80 ± 13
MgC20g	3.7	8 ± 0.4	50 ± 1	11 ± 0.5	63 ± 3	36 ± 1	9 ± 1	694 ± 42	82 ± 5
MgC20h	3.7	7 ± 0.5	48 ± 1	11 ± 0.5	66 ± 2	36 ± 1	8 ± 1	753 ± 62	93 ± 5
MgC20i	3.7	8 ± 0.5	54 ± 1	12 ± 0.3	71 ± 2	42 ± 1	9 ± 1	856 ± 76	96 ± 5
MgC20j	3.7	7 ± 0.4	51 ± 2	12 ± 0.4	67 ± 2	38 ± 1	8 ± 1	800 ± 49	103 ± 4
Iwa 1	3.7	6 ± 0.4	50 ± 1	11 ± 0.5	64 ± 2	37 ± 1	8 ± 1	890 ± 81	116 ± 9
Iwa 2	3.7	7 ± 0.4	51 ± 1	12 ± 0.3	66 ± 3	39 ± 2	8 ± 1	912 ± 88	117 ± 7
[a]F_s [d.f. = 11,119]	–	8.13***	8.35***	6.25***	5.42***	7.58***	3.72***	7.78***	11.17***
LSD (5%)	–	1	4	1	6	3	2	157	16

[a] F_s value from analysis of variance

*** Represents significant differences between means at the 0.1% probability level

Table 3 Mean values ± SE for eight quantitative traits from plants grown in a screen-house of the DG4 series of transgenic lines derived from the same original transformed cell, plus the non-transgenic control of the parental cultivar Iwa (n = 10)

Line	Number of shoots/plant	Plant height (cm)	Leaf length (cm)	Terminal leaflet length (mm)	Terminal leaflet width (mm)	Number of tubers/ plant	Total tuber weight/ plant (g)	Weight/ tuber (g)
DG4a	3 ± 0.2	39 ± 2	12 ± 0.1	38 ± 2	19 ± 1	5 ± 1	53 ± 3	12 ± 1
DG4b	5 ± 0.2	50 ± 2	11 ± 0.1	59 ± 2	40 ± 2	8 ± 1	556 ± 15	70 ± 3
DG4c	6 ± 0.2	62 ± 2	13 ± 0.2	72 ± 2	44 ± 2	9 ± 1	512 ± 11	60 ± 3
DG4d	7 ± 0.3	68 ± 1	16 ± 0.1	80 ± 3	48 ± 1	13 ± 1	647 ± 14	54 ± 4
DG4e	4 ± 0.3	48 ± 2	13 ± 0.1	46 ± 3	21 ± 1	5 ± 1	63 ± 5	13 ± 1
Iwa control	7 ± 0.3	72 ± 2	18 ± 0.1	84 ± 2	49 ± 1	9 ± 1	635 ± 10	76 ± 3
[a]F_s [d.f. = 5,59]	43.34***	66.29***	10.39***	115.69***	125.31***	37.55***	761.26***	121.10***
LSD (5%)	1	4	2	5	3	1	29	7

[a] F_s value from analysis of variance

*** Represents significant differences between means at the 0.1% probability level

The appearance of phenotypic off-types among transgenic potato lines evaluated in the field has also been commonly reported (e.g. Dale and McPartlan 1992; Jongedijk et al. 1992; Belknap et al. 1994; Conner et al. 1994; Davidson et al. 2002a, 2002b; Heeres et al. 2002; Conner 2007). Phenotypic variation among plants regenerated from tissue culture is generally considered to arise from both pre-existing somatic variation in the source tissue of the parent plant and variation induced during the tissue culture phase (Veilleux and Johnson 1998; Duncan 1997). The altered phenotype exhibited by transgenic plants is further complicated by the possibility of mutagenesis arising from transgene insertion (Conner and Christey 1994; Veilleux and Johnson 1998). Distinguishing between these possibilities has been difficult in clonal crops such as potato, due to their highly heterozygous background and the resulting inherent phenotypic variability associated with allele segregation (Howard 1970). In this study we have used transgene integration as a molecular tag of single somatic cells of potato after their introduction into cell culture in order to trace the origin of tissue-induced variation.

Agrobacterium-mediated transformation was used to introduce modified T-DNAs into potato leaf cells. Following the selection of transformed cells, multiple shoots were

Table 4 Mean values ± SE for eight quantitative traits from plants grown in a screen-house of the MgC20 series of transgenic lines derived from the same original transformed cell, plus the non-transgenic control of the parental cultivar Iwa (n = 10)

Line	Number of shoots/plant	Plant height (cm)	Leaf length (cm)	Terminal leaflet length (mm)	Terminal leaflet width (mm)	Number of tubers/ plant	Total tuber weight/ plant (g)	Weight/ tuber (g)
MgC20a	6 ± 0.3	53 ± 1	16 ± 0.6	77 ± 2	48 ± 1	7 ± 1	542 ± 14	75 ± 2
MgC20b	7 ± 0.2	52 ± 1	15 ± 0.6	67 ± 1	39 ± 1	11 ± 1	552 ± 19	51 ± 3
MgC20c	6 ± 0.4	49 ± 1	14 ± 0.7	69 ± 2	40 ± 2	9 ± 1	556 ± 14	64 ± 2
MgC20d	6 ± 0.3	56 ± 1	15 ± 0.6	70 ± 2	38 ± 1	10 ± 1	613 ± 14	65 ± 2
MgC20e	4 ± 0.3	45 ± 2	10 ± 0.4	57 ± 2	31 ± 1	6 ± 1	242 ± 16	42 ± 2
MgC20f	7 ± 0.3	46 ± 1	12 ± 0.5	62 ± 2	38 ± 2	9 ± 1	485 ± 18	58 ± 2
MgC20g	8 ± 0.3	56 ± 1	13 ± 0.4	70 ± 2	41 ± 1	9 ± 1	595 ± 15	68 ± 2
MgC20h	7 ± 0.3	65 ± 1	13 ± 0.6	69 ± 2	39 ± 2	10 ± 1	630 ± 15	66 ± 2
MgC20i	8 ± 0.2	70 ± 2	17 ± 0.5	79 ± 2	51 ± 1	9 ± 1	658 ± 11	75 ± 2
MgC20j	7 ± 0.3	63 ± 1	15 ± 0.4	71 ± 2	47 ± 2	9 ± 1	639 ± 9	73 ± 3
Iwa control	7 ± 0.3	72 ± 2	18 ± 0.5	88 ± 2	51 ± 1	9 ± 1	630 ± 14	75 ± 2
[a]F_s [d.f. = 10,109]	17.20***	41.72***	21.21***	29.22***	30.77***	10.10***	67.85***	25.89***
LSD (5%)	1	4	1	4	3	1	40	6

[a] F_s value from analysis of variance

*** Represents significant differences between means at the 0.1% probability level

regenerated from individual cell colonies and Southern analysis on fragments surrounding the integration site was used to confirm their derivation from a single transformed cell (e.g. Figs. 1a, 2a). In this manner several series of transgenic potato lines were produced, each consisting of multiple regeneration events derived from the same transformed cell. Clonally multiplied plants from each regeneration event were established in a field trial for assessment of eight quantitative traits. Analysis of phenotypic variation in these traits demonstrated highly uniform plants within the clones from each regeneration event, but markedly different between the independent regeneration events (Figs. 1b, 2b, Tables 1, 2, 3 and 4). Such results have been observed following transformation of four different potato cultivars and different binary vectors with a *cry* gene or an antibacterial magainin gene. Analysis of nuclear DNA content by flow cytometry established that all the transgenic lines regenerated from the same cell had an identical DNA content (Tables 1, 2), equivalent to the original potato cultivar. This establishes that the observed phenotypic variation was not a consequence of ploidy or gross aneuploidy alterations.

If the origin of this phenotypic variation was pre-existing in the somatic tissue of the parent plant, then all regenerated potato lines derived from a single cell would have the same phenotype. The observed phenotypic variation between these lines must have occurred after T-DNA insertion, and therefore during the tissue culture phase after the cell was introduced into culture. Since a molecular marker has been used to tag the clonal origin of single somatic cells in tissue culture, this result unequivocally demonstrates for the first time that somaclonal variation occurs during tissue culture, and that it arises independently of transgene insertion. Although somatic variation is generally assumed to arise in cell and tissue culture, prior evidence has been based on the association between longer periods in culture and higher frequencies of variation, and variation being observed between plants assumed to have regenerated from the same cell.

More importantly, this study also established that the first shoots recovered do not necessarily exhibit less somaclonal variation, since later regeneration events can give rise to plants that are more phenotypically normal. For example, among the DG4 series the first and fifth shoots regenerated produced plants that exhibited very poor performance, whereas the second to fourth shoots regenerated produced substantially higher performing plants (Tables 1, 3). This result has important implications for the recovery of phenotypically normal transgenic lines for the genetic improvement of clonal crops. Similarly, in the MgC20 series the fifth regenerated shoot produced very poorly performing plants, whereas the eighth to tenth shoots were among the highest performing lines (Tables 2, 4).

The usual approach for producing transgenic cultivars in clonal crops involves the development of efficient transformation systems, then the development of a large number of independently selected transgenic lines in order to recover several lines with the desired level of transgene expression, as well as a phenotypically normal appearance and yield under field conditions (Conner and Christey 1994; Conner 2007). This is essential in clonal cultivars which are highly heterozygous with considerable inbreeding depression. Further

breeding to eliminate tissue-culture induced variation is undesirable since the genetic integrity of elite clones is immediately lost upon selfing or outcrossing (Howard 1970; Simmonds 1979). When developing large populations of independently derived transgenic lines, the first shoot to regenerate from each transformation event is usually selected. This is based on a widely held premise that the frequency of somaclonal variation increases with longer periods in cell culture, and the resulting assumption that the longer time a shoot takes to regenerate, the greater chance that an 'off-type' will appear (Conner 2007). This study has established that this assumption is not necessarily correct, since the most phenotypically normal plants do not necessarily arise from the first shoot regenerated from a transformed cell. Consequently, a new strategy is proposed to facilitate the recovery of phenotypically normal transgenic lines in clonal crops such as potatoes, yams, sugarcane, fruit trees and many other fruit crops, as well as some forestry and ornamental species. When developing transgenic lines for genetic improvement of clonal crops, multiple shoots should be regenerated and evaluated from each transformation event, rather than single shoots from very large populations of transformation events.

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References

Barrell PJ, Conner AJ (2009) Expression of a chimeric magainin gene in potato confers improved resistance to the phytopathogen Erwinia carotovora. Open Plant Sci J 3:14–21

Barrell PJ, Shang YJ, Cooper PA, Conner AJ (2002) Alternative selectable markers for potato transformation using minimal T-DNA vectors. Plant Cell Tiss Organ Cult 70:61–68

Belknap WR, Corsini D, Pavek JJ, Snyder GW, Rockhold R and Vayda, ME (1994) Field performance of transgenic Russet Burbank and Lemhi Russet potatoes. Am Potato J 71:285–296

Conner AJ (2007) Field-testing of transgenic potatoes. In: Vreugdenhil D (ed) Potato biology, biotechnology: advances, perspectives. Elsevier, Amsterdam, pp 687–703

Conner AJ, Christey MC (1994) Plant breeding and seed marketing options for the introduction of transgenic insect-resistant crops. Biocontrol Sci Tech 4:463–473

Conner AJ, Williams MK, Abernethy DJ, Fletcher PJ, Genet RA (1994) Field performance of transgenic potatoes. N Z J Crop Hort Sci 22:361–371

Dale PJ, McPartlan HC (1992) Field performance of transgenic potato plants compared with controls regenerated from tuber discs and shoot cuttings. Theor Appl Genet 84:585–591

Davidson MM, Jacobs JME, Reader JK, Butler RC, Frater CM, Markwick NP, Wratten SD, Conner AJ (2002a) Development and evaluation of potatoes transgenic for a *cry*1Ac9 gene conferring resistance to potato tuber moth. J Am Soc Hort Sci 127:590–596

Davidson MM, Takla MFG, Reader JK, Butler RC, Wratten SD, Conner AJ (2002b) Evaluation of field grown potato lines transgenic for a *cry*1Ac9 gene conferring resistance to potato tuber moth. N Z Plant Prot 55:405–410

Deroles SC, Gardner RC (1988) Expression and inheritance of kanamycin resistance in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. Plant Mol Biol 11:355–364

Duncan RR (1997) Tissue culture-induced variation and crop improvement. Adv Agron 58:201–240

Gleave AP, Mitra DS, Markwick NP, Morris BAM, Beuning LL (1998) Enhanced expression of the *Bacillus thuringiensis cry*9Aa2 gene in transgenic plants by nucleotide sequence modification confers resistance to potato tuber moth. Mol Breed 4:459–472

Heeres P, Schippers-Rozenboom M, Jacobsen E, Visser RGF (2002) Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variation. Euphytica 124:13–22

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218

Howard HW (1970) Genetics of the potato-*Solanum tuberosum*. Logos Press, London

Jongedijk E, de Schutter AAJM, Stolte T, van den Elzen PJM, Cornelissen BJC (1992) Increased resistance to potato virus X and preservation of cultivar properties in transgenic potatoes under field conditions. Bio/Technology 10:422–429

Karp A (1991) On the current understanding of somaclonal variation. Oxford Surv Plant Mol Cell Biol 7:1–58

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

Morgan ER, Burge GK, Seelye JF, Grant JE, Hopping ME (1995) Interspecific hybridisation between *Limonium perigrinum* Bergius and *Limonium purpuratum* L. Euphytica 83:215–224

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarium 15:473–497

Phillips RL, Kaeppler SM and Olhoft P (1994) Genetic instability of plant tissue cultures: Breakdown of normal controls. In: Proceedings of the National Academy of Sciences (USA) 91:5222–5226

Potter R, Jones MGK (1991) An assessment of genetic stability of potato in vitro by molecular and phenotypic analysis. Plant Sci 76:239–248

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Secor GA, Shepard JF (1981) Variability of protoplast-derived potato clones. Crop Sci 21:102–105

Shepard JF, Bindey D, Shahin E (1980) Potato protoplasts in crop improvement. Science 208:17–24

Simmonds NW (1979) Principles of plant breeding. Longman, London

Sokal RR, Rolf FJ (1969) Biometry: the principles and practices of statistics in biological research. Freeman, San Francisco

Thieme R, Griess H (2005) Somaclonal variation in tuber traits of potato. Potato Research 48:153–165

Van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A, Stiekema WJ (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res 4:288–290

Veilleux RE, Johnson AAT (1998) Somaclonal variation: molecular analysis, transformation interaction, and utilization. Plant Breed Rev 16:229–268