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Transformation of diploid potato genotypes through *Agrobacterium* vectors and expression of T-DNA markers in root clones, regenerated plants and suspension cells

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SUMMARY

Stem internodes of three diploid potato genotypes (lines HH260, C and E) were transformed with binary *Agrobacterium* strains, containing the *A. rhizogenes* wild type plasmid pRi1855 together with the plasmid construct pBI121, to introduce a set of selectable and reporter marker genes (coding for hairy root phenotype, hormone autotrophy, opine synthesis, kanamycin resistance and β-glucuronidase activity). Genetic marker lines were produced at the level of root clone, plant (regenerated from root clone segments) and cell suspension culture (established from callus induced on leaf segments). Transformation frequencies and the expression of transformation marker characters were dependent on the genotype, the physiological state of the internodal explants and the *Agrobacterium* strain. Root clones, derived from stem internodes producing transformed roots in high numbers, generally showed a complete set of marker characters and prolific growth, in contrast to the root clones that originated from less productive stem internodes. Shoot regeneration was achieved from the genotypes HH260 and C, but not from genotype E. Loss of one or more marker characters (opine synthesis, kanamycin resistance, GUS activity) was observed in half of the regenerants, as compared to their original root clones. Cell suspension cultures showed expression of all marker characters present in the original transformed plant. The majority of the transformed marker lines, at the level of root clone, regenerated plant, or cell suspension culture, had maintained the original diploid level.

Key-words: Agrobacterium, binary vectors, ploidy stability, potato, transformation.

INTRODUCTION

Genetic markers are essential tools for selection and recognition in various somatic cell genetic investigations directed towards gene mapping and gene transfer, and so are useful for plant breeding. Biochemical variants (mutants) at the cellular level seemed promising

in this respect (Negrutiu *et al.* 1989). However, polyploidization, aneuploidy and chromosome rearrangements often occur *in vitro* (Pijnacker & Sree Ramulu 1990), which greatly limit the usefulness of the variant cell lines in somatic cell genetics. In recent years it became possible to introduce, through *Agrobacterium* transformation, well-defined selectable and reporter marker genes into potato. Transformation with hairy root-inducing binary vector strains of *Agrobacterium* is particularly useful for the production of genetic marker lines because of several advantages, i.e. single-cell origin of transformed roots (Tepfer 1984), high genetic stability of transformants (De Vries-Uijtewaal *et al.* 1989; Ottaviani *et al.* 1990), and integration of a single or a few copies of the three T-DNAs per genome at many possible loci spread over the different chromosomes (Ambros *et al.* 1986; Visser *et al.* 1989).

The present article reports data on transformation with two binary vector strains (LBA 1060KG and LBA 9402KG) of *Agrobacterium* in diploid potato genotypes C and E and in an interdihaploid line HH260. Selection of these genotypes was based on several characteristics that are useful for gene mapping and somatic cell genetics. The former two genotypes carry various morphological and isozyme markers (Jongedijk & Ramanna 1989), and the latter genotype exhibits high transformation frequency and high ploidy stability among the hairy root clones and regenerated plants (De Vries-Uijtewaal *et al.* 1988, 1989). In addition, results are presented on the expression of the introduced genetic marker characters in various transformed marker lines at the level of root clones, regenerated plants and cell suspension cultures. At these levels, the genetic marker lines have specific advantages in gene transfer and gene mapping experiments.

MATERIALS AND METHODS

Plant material

For transformation experiments the following genotypes of *Solanum* were used: line HH260 (interdihaploid, *S. tuberosum*, $2n=2x=24$) (Binding *et al.* 1978); line C (USW5337-3, *S. tuberosum* × *S. phureja*, $2n=2x=24$), and line E (77.2102-37, $2n=2x=24$) obtained from the cross USW5337-3 (=line C) × $VH^3$4211 (V=*S. vernei*; H=*S. tuberosum*). The lines C and E carry several morphological (desynapsis, crumpled, yellow cotyledon, flower colour, stigma colour, flesh colour), isozyme (GOT1, GOT2, MDH1, PGDH) and resistance markers (resistances against virus Y, *Phytophthora* and potato leaf rol virus) (Jongedijk & Ramanna 1988, 1989, Jongedijk *et al.* 1990). The genotypes were multiplied monthly *in vitro* as shoot cultures under controlled conditions of 16 h d^{-1} light (TL type FTD58W33) and 24°C in glass jars on solid MS medium (Murashige & Skoog 1962) supplemented with 2% sucrose and 0·8% agar (Oxoid) (De Vries-Uijtewaal *et al.* 1988).

Bacterial strains

The following two strains of *Agrobacterium*, both containing pRi1855 and pBI121 (Fig. 1), were used for transformation: *A. tumefaciens* strain LBA 1060KG (De Vries-Uijtewaal *et al.* 1989) and *A. rhizogenes* strain LBA 9402KG. The latter was produced by M.F. van Wordragen (Centre for Plant Breeding Research CPO, Wageningen, The Netherlands) through the introduction of the construct pBI121 into *A. rhizogenes* strain LBA 9402 (Spanò *et al.* 1982).

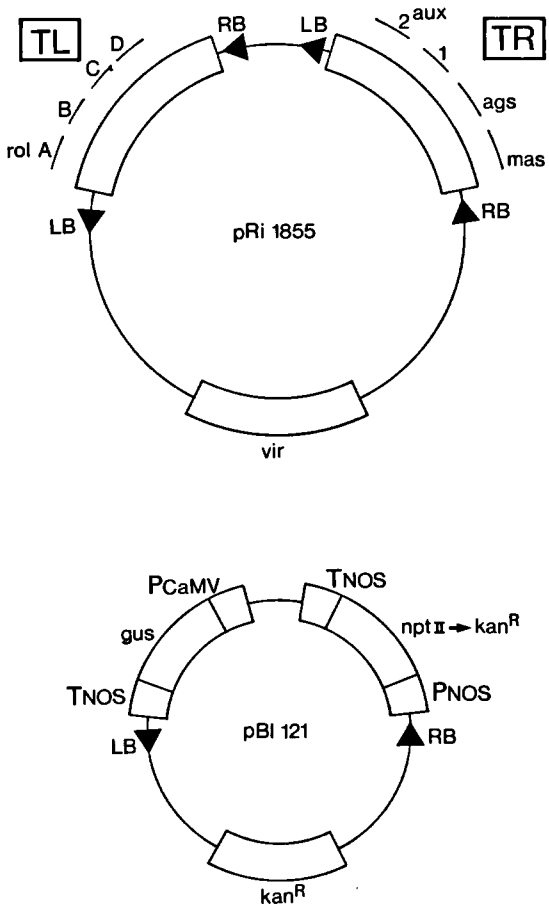

Fig. 1. Schematic diagram of the *Agrobacterium* strains LBA 1060KG, and LBA 9402KG containing pRi1855 and pBI121. The plasmid pRi1855 carries two independent T-DNAs (Cardarelli *et al.* 1985): the TL-DNA coding for the hairy root phenotype and hormone autotrophy (*rol* loci), and the TR-DNA coding for auxin synthesis (*aux* 1,2), and agropine (*ags*) and mannopine (*mas*) synthesis. The binary vector pBI121 contains the neomycin phosphotransferase (*npt* II) gene (responsible for kanamycin resistance) fused to the nopaline synthase promoter and terminator, together with the *E. coli* β-glucuronidase (GUS) gene fused to the CAMV 35S promoter and nopaline synthase terminator (Jefferson *et al.* 1986, 1987). The promoter and terminator DNA-sequences enable expression of the genes in higher plants. The *vir* region of pRi1855 is responsible for transfer of the T-DNAs of both plasmids, whereas the *kan*^{R} region of the pBI enables stable maintenance of the plasmid in the bacteria when cultured on kanamycin containing medium.

Transformation and root clone culture

Stem internodes, inoculated with *Agrobacterium* strain LBA 1060KG, or LBA 9402KG, were placed on solidified hormone-free MS medium containing 20 $g\,l^{-1}$ sucrose, 350 $mg\,l^{-1}$ cefotaxim, and 100 mg l^{-1} kanamycin to select for hormone-autotrophic, kanamycin-resistant primary transformed roots (for more details on the method of transformation, see De Vries-Uijtewaal *et al.* 1988). The frequency of transformation is defined as the percentage of stem internodes that form one or more primary hairy roots within 3 weeks after inoculation. Three to 4-week-old primary roots were isolated and cultured as individual root clones on hormone-free MS medium without kanamycin at 24°C in darkness. Root clones were subcultured monthly.

Table 1. Expression of various genetic marker characters in root clones of the potato genotypes HH260, C and E obtained after transformation with *Agrobacterium* strain LBA 1060KG

Genotypes	Number of root clones tested	Number of root clones with		
		Opine$^+$	Kan^R	GUS$^+$
HH260	9	7	9	5
C	7	7	7	5
E	28*	26	28	25
	18‡§	5	17	15
Total	62	45	61	50

*Root clones from stem internodes that produced eight or more transformed roots.
‡Root clones from stem internodes that produced seven or less transformed roots.
§Two of these root clones showed absence of both opine synthesis and GUS activity.
Opine$^+$ = synthesis of agropine and mannopine.
Kan^R = resistance to 100 mg/l kanamycin.
GUS$^+$ = β-glucuronidase activity.

Plant regeneration

To induce shoot regeneration, segments of root clones with a length of 1–2 cm were cultured under controlled conditions (24°C, 16 h d^{-1} light of 1 klx) on solid MS media with various hormone and sucrose concentrations as reported in tables 1 and 3 in De Vries-Uijtewaal *et al.* (1989). Regenerated plants were subcultured monthly on hormone-free MS medium without kanamycin under the culture conditions as mentioned above.

Cell suspension cultures

Cell suspension cultures were established from calli which developed on leaf segments of the transformed regenerants placed on MS medium containing 20 g l^{-1} sucrose, 5 mg l^{-1} NAA and 1 mg l^{-1} BAP. Suspension cells were cultured in darkness in MS medium, containing 20 g l^{-1} sucrose, 5 mg l^{-1} NAA and 0·1 mg BAP, and subcultured weekly.

Characterization of root clones, regenerated plants and suspension cells

The expression of hormone autotrophy by root clones and cell suspensions was revealed during culturing on the media without hormones. The presence of agropine and mannopine was determined by paper electrophoresis and silver staining according to Petit *et al.* (1983). Kanamycin resistance was tested by culturing in the respective media to which 100 mg l^{-1} kanamycin was added. The activity of GUS was assayed by fluorimetry (Jefferson *et al.* 1986, 1987). Ploidy levels were determined by chromosome counts using the Feulgen technique, and by flow cytometric determination of the DNA content of isolated interphase nuclei (Sree Ramulu & Dijkhuis 1986).

RESULTS

The effects of genotype, explant age and *Agrobacterium* strain on the frequency of transformation were investigated. The transformation frequencies obtained after inoculation of stem internodes from 3-week-old shoot cultures with *Agrobacterium* strain

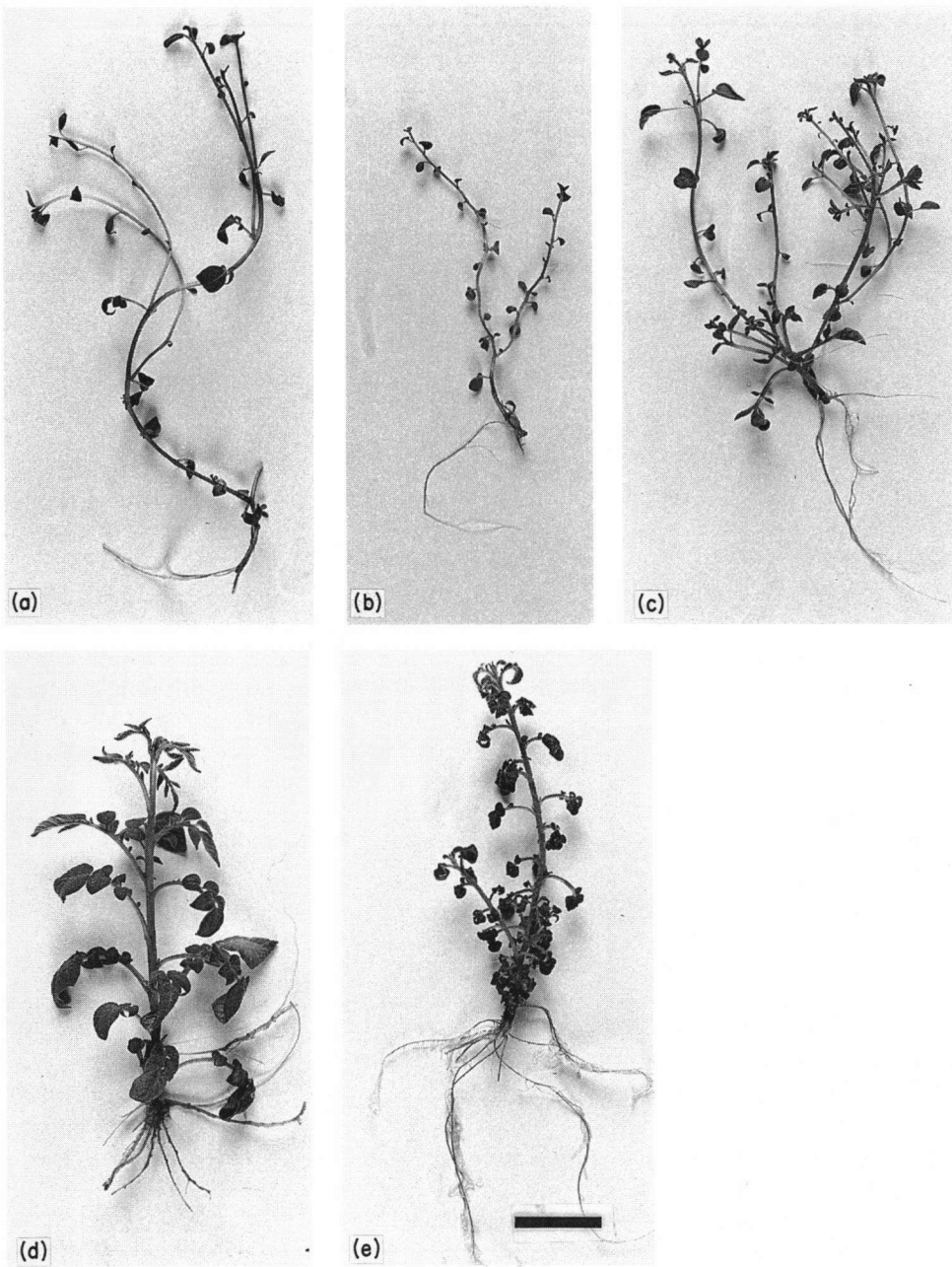

Fig. 2. Phenotypes of in-vitro grown 3-week-old plantlets of the diploid potato genotypes HH260, C and E ($2n = 2x = 24$): Figs 2a–c, respectively, and a transformed regenerant with representative phenotype of HH260 and C: Figs 2d–e, respectively. Bar represents 1 cm.

Table 2. Opine (agropine and mannopine) synthesis, kanamycin resistance (Kan^R), β-glucuronidase activity (GUS), and ploidy level of root clones (RC), regenerated plants (PL) and cell suspension cultures (SC) in two diploid potato genotypes

Genotypes	Clones	Stage	Opine	Kan^R	GUS	Ploidy
HH260	357	RC	−	+	+	2*x*
		PL	−	+	+	2*x*
		SC	−	+	+	2*x*
	359	RC	+	+	−	2*x*
		PL	+	+	−	2*x*
		SC	+	+	−	2*x*
	360	RC	+	+	−	2*x*
		PL	+	−	−	4*x*
		SC	ND	ND	−	ND
	361	RC	+	+	−	2*x*
		PL	+	+	−	2*x*/4*x*
		SC	+	+	−	ND
	362	RC	+	+	−	2*x*
		PL	+	+	−	2*x*
		SC	+	+	−	2*x*
	366	RC	+	+	+	2*x*
		PL	−	+	+	2*x*
		SC	−	+	+	2*x*
	403	RC	+	+	+	2*x*
		PL	−	−	+	2*x*
		SC	−	−	+	ND
	413	RC	+	+	+	2*x*
		PL	+	+	+	2*x*
		SC	+	+	+	2*x*
C	C1	RC	+	+	+	2*x*
		PL	−	+	+	2*x*
		SC	−	+	+	2*x*
	C2	RC	+	+	+	2*x*
		PL	+	−	+	2*x*
		SC	ND	ND	ND	ND
	C4	RC	+	+	+	2*x*
		PL	+	−	+	4*x*
		SC	ND	ND	ND	ND
	C101	RC	−	+	+	2*x*
		PL	−	+	+	2*x*
		SC	−	+	+	2*x*/4*x*

ND = Not determined.

LBA 1060KG were 35% ($n = 32$ stem internodes) for genotype HH260, 23% ($n = 130$) for genotype E and only 3% ($n = 105$) for genotype C. Inoculation with strain LBA 9402KG resulted in higher frequencies of transformation, i.e. 63% ($n = 19$) for genotype E and 14%

($n = 21$) for genotype C (not determined for genotype HH260). The genotype C responded with increased transformation frequency (28%; $n = 36$) when stem internodes from much older (11-week-old) shoot cultures were used for inoculation with LBA 9402KG.

The expression of the different marker characters was analysed in several root clones of the genotypes HH260, C and E after prolonged culture (approximately 6 months) in the absence of hormones and kanamycin (i.e. only maintaining selection pressure for hormone autotrophy) (Table 1). In HH260, two of the nine root clones lacked opine synthesis, while GUS-activity was absent in four other root cones. In the genotype C, two root clones did not show GUS-activity. In genotype E, 28 root clones were obtained from stem internodes that produced eight or more transformed roots, and 18 root clones from stem internodes which gave rise to seven or less transformed roots. The number of root clones with expression of all the three marker characters was much higher in the former than in the latter category where many root clones lacked opine synthesis. Root clones of genotype E that expressed all the three marker characters, also showed in general, profuse, hormone autotrophic growth and hairy root phenotype.

Plant regeneration was obtained from approximately 50% of the root clones of the genotypes HH260 (8 out of 16) and C (10 out of 22). No regeneration was obtained from root clone segments of genotype E. When compared to the parental plants, the transformed regenerants showed more vigorous growth and compound leaves (Fig. 2). The leaves of the regenerants of genotype C were small and crinkled. Leaf segments of all regenerants gave rise to calli which were used to establish cell suspension cultures.

Table 2 gives data on opine production, kanamycin resistance, GUS-activity, and the ploidy level of the root clones, their regenerants and cell suspension cultures. In all, 12 clones were analysed. Only the clone 413 contained the complete set of markers at all three levels. Of the other 11 clones, six root clones (357, 359, 360, 361, 362, C101) showed no expression of opine synthesis or GUS-activity. At the regenerated plant level six clones (360, 366, 403, C1, C2, C4) lost expression of one or more marker characters. On the other hand, cell suspension cultures derived from the regenerated plants showed no (further) loss of markers. Generally, the parental ploidy level (i.e. diploid) was maintained throughout transformation, regeneration and cell culture.

DISCUSSION

The results obtained show that the frequency of transformation, as well as the expression of transformation marker characters in the various root clones, were dependent on the plant genotype, the physiological state of the individual stem internodal explant and the *Agrobacterium* strain. It is known that these factors can strongly influence the integration of T-DNA and its transcriptional activation, e.g. by DNA methylation (Peerbolte 1986). Furthermore, when compared to kanamycin resistance (*npt* II gene), which was expressed in all the root clones (except one), GUS activity was absent in 12 out of 62 root clones. Similar results were obtained in the tetraploid potato cultivar 'Bintje' by Ottaviani and Hänisch ten Cate (in press). As both the *npt* II and GUS genes are placed between the same borders (Fig. 1), they are probably inserted at the same locus of the plant genome. Therefore, the frequent absence of GUS activity can be attributed to differential expression of the NOS and CAMV35S promoters.

The regeneration capacity was found to be high in root clones derived from the genotypes HH260 and C, whereas the root clones obtained from the genotype E failed to respond to various regeneration procedures used according to De Vries-Uijtewaal *et al.*

(1989). As the genotype E was obtained from the cross USW5337-3 (C = *S. tuberosum* × *S. phureja*) × VH[3]4211 (V = *S. vernei*, H = *S. tuberosum*) (Jongedijk & Ramanna 1988), half of the genome of line E is equal to that of C, while the other half is derived from *S. vernei* and *S. tuberosum*. Therefore, the recalcitrance to plant regeneration under the present experimental conditions might be ascribed to the *S. vernei* genome part, or to the genotypic complexity.

With regard to the expression of marker genes at regenerated plant level, in comparison to the original root clones, 50% of the plants showed the loss of kanamycin resistance or opine synthesis, the majority of which occurred independently, i.e. in different plants. In contrast, in cell suspensions no (further) loss of marker genes occurred, althouth suspension culture was established from callus. Deletion of the introduced alien genes has been observed frequently among regenerated plants (Peerbolte 1986; Hänisch ten Cate *et al.* 1990). Although ploidy stability after Ri T-DNA transformation is generally maintained (see also de Vries-Uijtewaal *et al.* 1988, 1989), the occurrence, during the plant regeneration process and plant development of cryptic chromosomal or gene rearrangements leading to the deletion or inactivation of introduced genes, cannot be excluded.

Various transformed lines at the level of regenerated plants and cell suspensions, produced in this study, are being currently used for gene mapping and partial genome transfer (Gilissen *et al.* 1989; Sree Ramulu *et al.* 1990a,b).

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