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Fate of introduced genetic markers in transformed root clones and regenerated plants of monohaploid and diploid potato genotypes

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Summary. *Agrobacterium* transformation of stem internodes of four monohaploid (839-79, 849-7, 851-23, 855-1) and two diploid (M9 and HH260) potato genotypes using hairy root-inducing single (LBA 1020, LBA 9365, LBA 9402) and binary (LBA 1060KG) vectors is reported. Various media and successive culture steps were tested for plant regeneration from different transformed root clones. The fate of introduced genetic markers in root clones and regenerated plants (hairy root phenotype, hormone autotrophy, opine production, kanamycin resistance, β-glucuronidase activity), the ploidy stability and protoplast yield were analysed. The transformation efficiency of stem internodes (hairy root production) and the regeneration capacity of the transformed root clones greatly differed within and between the various potato genotypes. The regenerated plants obtained after transformation with both types of vectors often showed the absence of one or more genetic markers. However, transformation with the binary *Agrobacterium* vector generally resulted in the stable presence of the opines in all transformed root clones and most regenerated plants. In HH260, transformation efficiency, plant regeneration of transformed root clones, protoplast yield and ploidy stability were the highest as compared to the other genotypes. The application of these transformed plants as marker lines in gene mapping and gene expression studies is indicated.

Key words: Potato – *Agrobacterium* transformation – Plant regeneration – Monohaploid, diploid – Genetic markers

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Introduction

In potato, gene mapping by means of classical genetic methods is greatly hindered by the heterozygosity of the cultivars, by autotetraploidy and by sterility. In addition, there is a great scarcity of genetic markers. Until now only four genes controlling morphological characters (Lam and Erickson 1971; Hermsen et al. 1973; Wagenvoort 1982, 1988) and one gene controlling glucosylation of rutin (Lee and Row 1975) have been localized, while no molecular markers have been mapped yet. The use of genetic markers in somatic cell genetic and molecular biological investigations can considerably accelerate gene mapping in this species.

Genetic markers can be introduced into potato by means of transformation with *Agrobacterium* plasmids (Ooms et al. 1987; de Vries-Uijtewaal et al. 1988; Knapp et al. 1988; Sheerman and Bevan 1988; Stiekema et al. 1988). There is evidence that insertions of the T-DNA can take place at several loci spread over different chromosomes. Often, only one or a few copies of the T-DNA integrate into the host cell genome (Ambros et al. 1986; Stiekema et al. 1988). This enables one or a few chromosomes per cell to be tagged. In this regard, transformation by hairy root-inducing strains of *Agrobacterium* has several advantages: (1) Each root clone in general originates from one transformed cell (Tepfer 1984). (2) Root clones and plants regenerated from them show high stability in ploidy levels (Hänisch ten Cate et al. 1987, 1988). Hence, a series of root clones and plant lines, each containing a T-DNA copy inserted into a different chromosome, can be established. (3) Root clones can be selected for hormone autotrophy, which serves as a selectable marker (Gleba et al. 1986; Müller-Gensert and Schieder 1987). (4) Ri T-DNA is useful in binary vector systems to introduce other genetic markers, e.g. kanamycine resis-

tance (Shahin et al. 1986; Trulson et al. 1986; Sukhapinda et al. 1987).

Monohaploid and diploid potato lines are potentially useful for breeding and fundamental research (Wenzel et al. 1979; Uijtewaal 1987). For gene mapping also, genetic marker lines with low ploidy levels are highly desirable. So far, only two reports on transformation with *Agrobacterium* vectors in a diploid potato genotype (Knapp et al. 1988) and in monohaploid and diploid potato genotypes (de Vries-Uijtewaal et al. 1988) have been published. In the former study the authors reported, while comparing direct DNA transfer into protoplasts and leaf disc transformation via *Agrobacterium tumefaciens*, that the latter method resulted in high transformation frequency and genetic stability of transformed plants. De Vries-Uijtewaal et al. (1988) have characterized several root clones obtained after transformation of monohaploid and diploid potato genotypes with hairy root-inducing (single) vectors. In this paper, results are presented on *Agrobacterium*-mediated transformation using both single and binary hairy root-inducing vectors in four monohaploid and two diploid potato genotypes. The fate of the introduced genetic markers in hairy root clones and regenerated plants has been analysed.

Materials and methods

Plant material

For transformation experiments, the following genotypes of *Solanum* were used: the diploids ($2n=2x=24$) HH260 (interdihaploid) (supplied by the Max Planck Institut für Züchtungsforschung, Köln, BRD) (Binding et al. 1978) and M9 (provided by Prof. J.G.Th. Hermsen, Agricultural University Wageningen, The Netherlands); the monohaploids ($2n=x=12$) 839-79, 849-7, 851-23 and 855-1 (supplied by Dr. B.A. Uijtewaal, Agricultural University Wageningen, The Netherlands) (Uijtewaal et al. 1987). These genotypes were selected because of high production of hairy root clones after transformation of stem internodes with *Agrobacterium* Ri plasmids (de Vries-Uijtewaal et al. 1988). The genotypes were cultured in vitro as described previously (de Vries-Uijtewaal et al. 1988).

Bacterial strains

The following *Agrobacterium* strains were used in the transformation experiments:

(1) *A. rhizogenes* strain LBA 9365 (Chilton et al. 1982), containing pRi 8196 with the genes responsible for the hairy root phenotype, hormone autotrophy and the production of mannopine on a single T-DNA.

(2) *A. rhizogenes* strain LBA 9402 (Spanò et al. 1982) carrying pRi 1855 with two independent T-DNA's (Cardarelli et al. 1985): TL-DNA which codes for the hairy root phenotype and hormone autotrophy, and TR-DNA which codes for agropine and mannopine synthesis.

(3) *A. tumefaciens* strain LBA 1020 (=strain LBA 285+pRi 1855::Tn5) (Hooykaas et al. 1982): The plasmid of this strain contains the same TL- and TR-DNA as strain LBA 9402.

(4) *A. tumefaciens* strain LBA 1060KG. This strain is a binary vector derived from strain LBA 1060 (supplied by Dr. P.J.J. Hooykaas, State University Leiden, The Netherlands) containing pRi 1855 (see strain LBA 9402), to which the pBI 121 was added. The latter plasmid contains, between the T-DNA borders, the neomycine phosphotransferase gene fused to the nopaline synthase promoter and terminator, together with the *E. coli* β-glucuronidase gene fused to the CaMV 35S RNA promoter and nopaline synthase terminator (Jefferson et al. 1986, 1987).

(5) *A. tumefaciens* strain LBA 288 (Hooykaas et al. 1980), a cured strain used as a control.

Transformation and root clone culture

Stem internodes of in vitro cultured plants were used for transformation, essentially as described by de Vries-Uijtewaal et al. (1988). However, with regard to transformation by *A. tumefaciens* strain LBA 1060KG, stem internodes were placed on root culture medium (hormone-free MS medium containing 30 g/l sucrose + 350 mg/l cefotaxim: MS 30+cefo) (Murashige and Skoog 1962), supplemented with 100 mg/l kanamycin to select for hormone-autotrophic, kanamycin-resistant primary hairy roots. Isolated primary hairy roots, developed after transformation, were cultured as individual root clones on MS 30+cefo or on MS 30+cefo plus kanamycin (100 mg/l), respectively.

Plant regeneration

To induce shoot regeneration, segments of each root clone with a length of 1–2 cm were cultured under controlled conditions of 16 h/day light, 1 klx, and 24 °C, on solid MS media with varying hormone and sucrose concentrations (Table 1). In a single case, primary hairy roots were used directly for shoot regeneration. Shoots of approximately 1 cm were transferred to glass jars containing solid MS 20 medium with 20 g/l sucrose (MS 20) for rooting. Outgrowth of shoot initials could be stimulated by transferring the regenerating tissues to 10 ml of liquid, hormone-free MS 20 medium in a 50-ml Erlenmeyer flask, placed on a gyratory shaker (120 rpm, amplitude 25 cm) under the same controlled conditions as mentioned previously. From each root clone, the first regenerated shoot was used for further analysis.

Characterization of root clones and regenerants

All established root clones were cultured on hormone-free MS medium, thus showing hormone autotrophy. The presence of agropine and mannopine was determined by paper electrophoresis and silver staining, according to Petit et al. (1983).

Root clones after transformation with *A. tumefaciens* LBA 1060KG were subcultured monthly on solid MS 30 medium supplemented with 100 mg/l kanamycin, and were thus continuously selected for maintenance of the kanamycin resistance marker. During shoot regeneration, kanamycine was omitted from the media. Therefore, maintenance of kanamycin resistance in the regenerants had to be tested by analysing whether shoots of regenerants were able to produce roots growing into kanamycin (100 mg/l) containing medium.

The presence of β-glucuronidase (GUS) activity in the in vitro cultured root clones and regenerated plants was assayed by fluorimetry according to Stiekema et al. (1988). The age of the root clones and plants in vitro at the time of assay was between 3 and 5 weeks after subculture.

Ploidy levels of root clones and the regenerated plants were determined by counting the number of chromosomes, after Feulgen staining, in root tip metaphase cells (Sree Ramulu and Dijkhuis 1986) and by flow cytometric determination of nuclear DNA content of interphase cells (Hänisch ten Cate et al. 1986; Sree Ramulu and Dijkhuis 1986).

Table 1. Composition of various culture media (MS) used for shoot regeneration from hairy root clones of monohaploid and diploid genotypes of potato. Concentration of hormones in mg/l, and of sucrose in g/l

Culture media	Auxins			Cytokinins		ABA	GA3	Sucrose
	2,4-D	NAA	IAA	BAP	Zeatin			
RS	–	–	–	–	–	–	–	10, 15, 20
CI-1[a]	0.12	–	–	–	2.0	1.0	–	20
CI-2	0.05	–	–	–	1.0	–	–	20
SI-1[a]	–	–	–	2.25	–	–	10	30
SI-2	0.01	–	0.06	–	1.5	0.5	–	10
SI-3[b,c]	–	0.01	–	–	1.0	–	–	5
SE-1	–	–	–	1.5	–	–	0.01	20
SE-2	–	–	–	1.0	–	–	1.0, 3.0	20
SE-3[d]	–	–	1.0	1.0	–	–	0.3, 1.0, 3.0	10

RS – medium with reduced sucrose concentration, CI – callus induction medium, SI – shoot induction medium, SE – shoot elongation medium

[a] Ooms et al. (1985)

[b] Nitsch and Nitsch (1969)

[c] This medium was supplemented with mannitol (17 g/l) and vitamins

[d] Espinoza and Dodds (1985)

Table 2. Frequency of plants regenerated from root clones obtained after transformation of monohaploid and diploid potato genotypes with various *Agrobacterium* strains

Genotypes	Strains (LBA)	No. of root clones			
		Tested	With browning	With swollen green calli	With swollen green calli and shoot regeneration[a]
Monohaploids					
839-79	9402	1	0	1	0
849-7	1020	1	1	0	0
851-23	9402	3	1	2	0
855-1	9402	3	1	1	1
Diploids					
HH260	1020	7	7	0	0
	1060KG	16	5	3	8
	9365	5	3	0	2
	9402	9	5	0	3
	9402	2[b]	0	1	1
M9	1020	4	2	2	0
	9402	5	0	5	0

[a] For details of the regeneration procedure, see Table 3

[b] Primary hairy roots; one of these resulted in shoot regeneration (see clone 177 in Tables 3 and 4)

Results

Transformation and plant regeneration

Transformation of four monohaploid and two diploid genotypes of potato with various *Agrobacterium* strains resulted in the establishment of a series of root clones with hormone autotrophy. Eight root clones obtained from monohaploid genotypes, and 46 root clones and two primary hairy roots from diploid genotypes were tested for plant regeneration capacity (Table 2). Of these root clones, three from the monohaploid and 22 from the diploid genotypes produced friable, pale callus when the segments were cultured on callus-induction (CI) or shoot-induction (SI) media. These calli did not show further development and rapidly turned brown. The other root clone segments showed greening and swelling, and produced one or more compact, green calli. In some genotypes the green calli did not show further develop-

Table 3. Various culture media used for plant regeneration of root clones from different potato genotypes transformed with various *Agrobacterium* strains. Different durations (in days) of subculture (given in parentheses) indicate different responses of individual root clones

Genotypes	Strains used	Root clones Code no.	Culture media in successive subcultures[a]
Monohaploid			
855-1	9402	155	SI-3 (21) → SI-2 (33) → SE-1 (11)
Diploid			
HH260	9365	37	SI-1 (63) → SE-1 (47)
		39S	SI-1 (63) → SE-1 (47)
	9402	13	RS (41)
		76	SI-1 (50) → RS (13) → SI-2 (47) → SE-1 (21)
		107[b]	CI-1 (22) → SI-1 (34) → RS (27)
		177[c]	SI-1 (25) → SE-1 (68)
	1060KG	357	CI-2 (21) → SE-2 (21)
		359	RS (42)
		360	CI-2 (21) → SE-2[d]
		361	SE-3 (49)
		362	CI-2 (21) → SE-2[d]
		366	CI-2 (21) → SE-2[d]
		403	CI-2 (21) → SE-2[d]
		413	SE-3 (49)

[a] For details of the medium composition, see Table 1
[b] This root clone was obtained after *Agrobacterium* transformation of a tuber disc
[c] Primary hairy root
[d] Shoots appeared between 21 and 35 days after transfer to SE-2-medium
→ – Subculture

ment (839-79, 851-23, M9), whereas in others they frequently produced shoots (855-1, HH260) when cultured on shoot-induction or shoot-elongation (SE) media. Segments from root clones, obtained after transformation with *A. tumefaciens* strain LBA 1020, did not respond with shoot regeneration in any of the genotypes tested, although they were cultured under the same conditions as those of root clones transformed with the other *Agrobacterium* strains (see 849-7, HH260 and M9).

The individual root clones differed in their response to shoot regeneration on various types of culture media (Table 3). Two root clones (line 13 and 359) easily produced shoots with little or no intermediate callus when transferred to medium containing sucrose at 15 or 20 g/l. However, no shoots from both lines developed on the media CI and SI, although compact green calli were formed (results not given).

Six root clones (lines 107, 357, 360, 362, 366 and 403) regenerated shoots after callus induction followed by a shoot induction (line 107) or a shoot elongation period of 3–5 weeks (Table 3). Five root clones (lines 37, 39S, 76, 155 and 177) gave shoot regeneration when root segments were placed on SI medium, followed by transfer to SE medium. The root clones 361 and 413 produced shoots within 7 weeks from callus of root segments cultured on SE3 medium.

Characterization of root clones and regenerated plants from dihaploid HH260

Phenotype. The regenerated plants showed a more vigorous growth when compared to the mother plant, and had more compound leaves, except those of clone 107, which showed crinkled, single leaves (Fig. 1). The number of protoplasts isolated from 1 g of leaf material ranged from 1.0×10^6 to 7.7×10^6 (Table 4), which is similar to that of the untransformed control. However, in contrast with the control, protoplasts from transformed plants rarely showed sustained cell divisions, and hence very poor callus formation (results not shown). The roots of most of the regenerated plants showed the transformed phenotype, i.e. abundant growth with many branches and negative geotropism (Table 4).

Opine production. Among the transformants resulting from inoculation with *A. rhizogenes* strain LBA 9365, the root clone 37 and its regenerant showed a high amount of mannopine. Root clone 39S showed no detectable mannopine content, whereas the plant regenerated from it produced a low amount. Six out of 11 transformed root clones that resulted from infection with *A. rhizogenes* strain LBA 9402 showed agropine and mannopine production (de Vries-Uijtewaal et al. 1988). However, in the

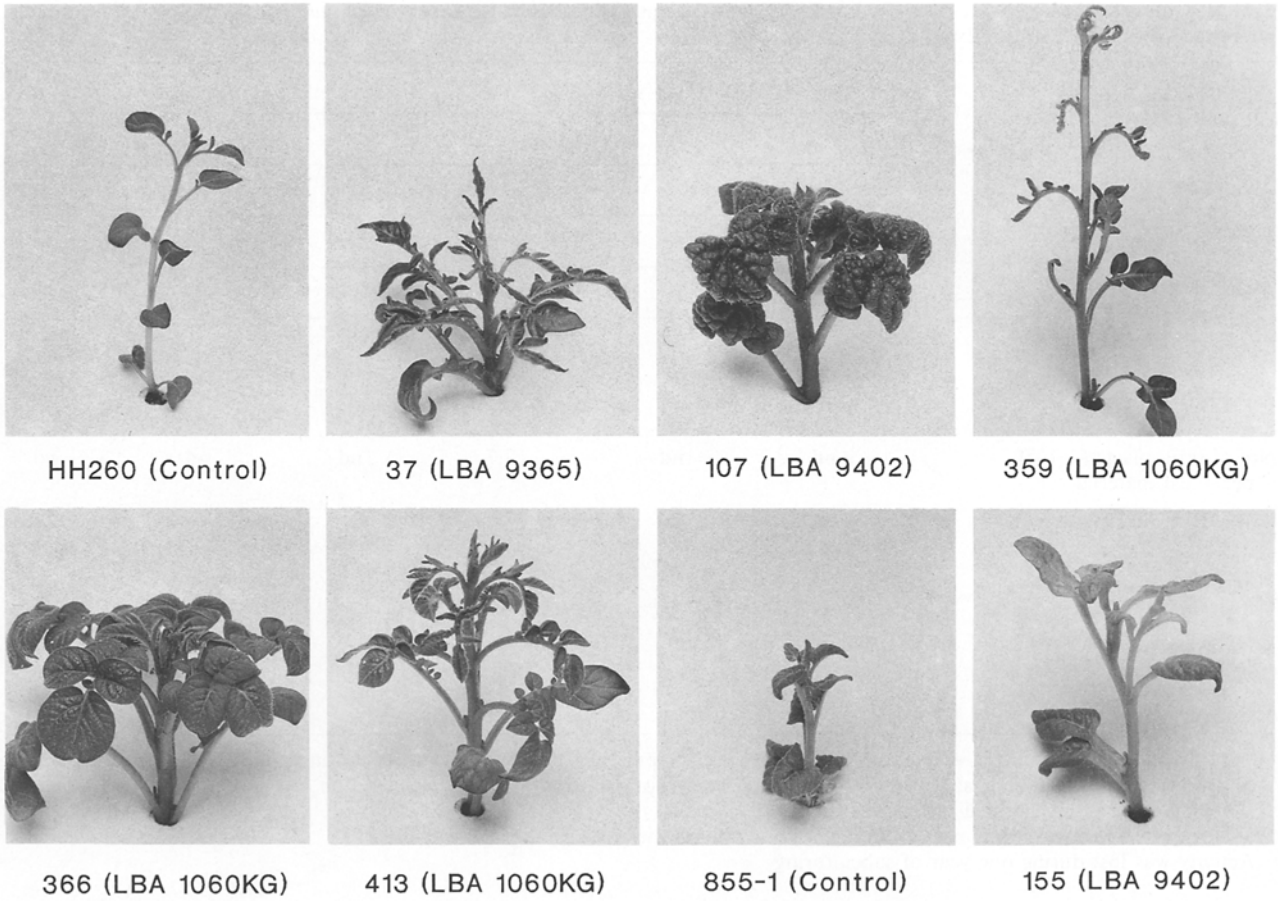

Fig. 1. Representative phenotypes of in vitro grown shoots regenerated from root clones obtained after transformation of the diploid potato genotype HH260 and the monohaploid genotype 855-1 with various strains of *Agrobacterium* as given in parentheses. The phenotypes of the lines 13, 177 (both from LBA 9402), 361, 362 and 403 (all from LBA 1060KG) were similar to that of line 413, and those of the lines 39S (from LBA 9365) and 76 (from LBA 9402) to that of 359

shoot-producing root clones (except in the regenerant of clone 76 with a low amount of mannopine), agropine as well as mannopine production were below the level of detection (Table 4). This is in contrast with the transformants from *A. tumefaciens* strain LBA 1060KG, in which both agropine and mannopine were found (average to high levels) in all the root clones and regenerants, except for the regenerants of the clones 366 and 403.

Ploidy levels. The ploidy level remained unchanged in all root clones and in the regenerants, except in the root clones 107 and 361. The clone 107 (obtained from tuber disc transformation) and its regenerant were tetraploid, and the clone 361 gave a regenerant with a mixoploid constitution (Table 4).

In order to investigate the influence of culture age on ploidy stability, 12 plants of line 13 were analysed, which were regenerated from the root clone during 6–36 weeks after transformation. The data showed that all plants had diploid chromosome numbers ($2n=24$) and were homogeneous in morphology and growth, all showing the transformed phenotype in the plant roots and shoots (results not given).

Kanamycin resistance and GUS activity. All root clones from the transformations with *A. tumefaciens* strain LBA 1060KG were resistant to kanamycin as determined from continuous culture on medium containing 100 mg/l kanamycin. The regenerant of the clone 403 had lost the resistance. Activity of β-glucuronidase was observed in all the root clones and regenerants except in the regenerants of the clones 359, 361 and 362 (Table 4).

Characterization of root clone and regenerated plant from the monohaploid 855-1

In contrast with diploid potato genotypes, the monohaploid 855-1 gave rise to diploid- or tetraploid-transformed root clones. One of its diploid root clones

Table 4. Characteristics of root clones (RC) and regenerated plants (PL) obtained after *Agrobacterium* transformation of the diploid potato genotype HH260 ($2n=2x=24$)

	Agrobacterium strain (LBA)											
	9365				9402							
	Potato line (code no.)											
	37		39S		13		76		107		177	
	RC	PL	RC	PL	RC	PL	RC	PL	RC	PL	RC[a]	PL
Phenotype of plant roots[b]												
growth rate		++		±		++		++		±		++
branching		±		−		++		++		±		++
geotropism		±		+		−		−		±		−
No. of protoplasts ($\times 10^6$) per g leaf material		nd		nd		7.7		nd		nd		nd
Ploidy level	2x	2x	2x	2x	2x	2x	2x	2x	4x	4x		2x
Opine production												
agropine[c]					−	−	nd	−	−	−		−
mannopine	++	++	−	±	−	−	nd	±	−	−		−
Kanamycin resistance (100 mg/l)												
β-glucuronidase act.												

−: not detected; ±: low level; +: average; ++: high level; p: present; a: absent; nd: not determined
[a] No root cone was available, since the entire primary hairy root was used for plant regeneration
[b] For phenotype of regenerated shoots, see Fig. 1
[c] T-DNA of *A. rhizogenes* strain LBA 9365 does not contain the genes for agropine synthesis
[d] Activity was lost during one year of subculturing

(line 155) (de Vries-Uijtewaal et al. 1988) gave regenerated plants that were also diploid. The cloned plant produced mannopine, but agropine was not detectable. The phenotype of the plant roots resembled that of the untransformed control. The shoots grew more vigorously than those of the mother genotype, but both formed single leaves (Fig. 1). The leaves of the transformants were, however, light green.

Discussion

The results show that the stem internode transformation of several monohaploid and diploid potato genotypes with single and binary hairy root-inducing *Agrobacterium* vectors gave rise to many root clones. The transformation efficiencies differed among the various potato genotypes as has been shown by de Vries-Uijtewaal et al. (1988). The transformed root clones were selected for hormone autotrophy when obtained after transformation with the single vectors, and in addition for kanamycin resistance in the case of transformation of the genotype HH260 with the binary vector. There was no difference in the frequency of transformation (i.e. primary hairy root production) with the single and the binary vectors in HH260 (result not shown).

With regard to plant regeneration, differences were observed among the root clones within and between the various potato genotypes. Three interdependent factors are probably involved: (1) the genotype, (2) the culture conditions, (3) the place(s) of integration and the copy number of the T-DNA's. In general, shoot regeneration was preceded by the formation of compact green callus. The ploidy level of plants, regenerated from this type of callus, in most cases remained the same as that of the original root clone. This is in agreement with the results on ploidy stability of plants regenerated from root clones of tetraploid genotypes (Hänisch ten Cate et al. 1988).

The marker genes (hairy root phenotype, hormone autotrophy, opine production, kanamycin resistance, β-glucuronidase activity) are located on physically separated T-DNA loci of the different *Agrobacterium* strains used. The TL-DNA codes for the hairy root phenotype and hormone autotrophy, the TR-DNA also for opine production, and the T-DNA of pBI 121 for kanamycin resistance and β-glucuronidase activity. In the case of transformation with the binary vector stain LBA 1060KG, opine production was more stably expressed, as

Table 4. Continued

	Agrobacterium strain (LBA)											
	1060KG											
	Potato line (code no.)											
	359		361		362		366		403		413	
	RC	PL	RC	PL	RC	PL	RC	PL	RC	PL	RC	PL
Phenotype of plant roots[b]												
growth rate		+		++		++		+		++		++
branching		+		+		+		+		+		+
geotropism		−		−		±		−		−		−
No. of protoplasts ($\times 10^6$) per g leaf material		3.3		3.7		nd		3.6		1.0		3.1
Ploidy level	2x	2x	2x	2x, 4x	2x	2x	2x	2x	2x	2x	2x	2x
Opine production												
agropine	++	+	++	+	++	+	±	−	++	−	++	+
mannopine	+	+	+	+	+	+	±	−	++	−	+	+
Kanamycin resistance (100 mg/l)	p	p	p	p	p	p	p	p	p	a	p	p
β-glucuronidase act.	p[d]	a	p[d]	a	p[d]	a	p	p	p	p	p	p

compared to that in the single vector *Agrobacterium* strain LBA 9402 (de Vries-Uijtewaal et al. 1988). The stable presence of the opines in the transformed root clones and regenerants obtained after transformation with the binary vector might be ascribed to tandem integration in the potato genome of the TL-, TR- and the vector (pBI 121) DNAs, as was also concluded from genetic analysis of the progeny of transformants in diploid potato (R.G.F. Visser, personal communication).

As concerned the expression of the other genetic marker characters (kanamycin resistance, β-glucuronidase activity, hairy root phenotype), the regenerated plants showed more often the absence of one or more of these characters (Table 4). This might be due to inactivation of T-DNA genes (Sinkar et al. 1988), probably because of DNA methylation (Gruenbaum et al. 1981), and/or due to partial deletion of T-DNA (Peerbolte 1986). Southern blot analysis will further distinguish between the occurrence of deletion and/or inactivation of the T-DNA genes, and in situ hybridization might reveal the loci of the host cell genome where the different T-DNA integrations have taken place.

Of all the potato genotypes studied, HH260 responded with high transformation frequency, plant regeneration from transformed root clones, protoplast yield from transformed plants and ploidy stability. The transformed lines carrying selectable markers are being used for the establishment of monochromosomal hybrids or addition lines through protoplast or microprotoplast fusion (Sree Ramulu et al. 1988; Verhoeven et al. 1988; Gilissen et al. 1989) and selection on kanamycin resistance and hormone autotrophy.

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Note added in proof

In the Introduction of this paper it has been mentioned that "Until now ... (in potato) no molecular markers have been mapped yet". However, recently a RFLP map of potato was published (Bonierbale et al. 1988, Genetics 120: 1095–1103) and another one is in preparation (Gebhardt et al. 1989, Vorträge für Pflanzenzüchtung 15: 29/6).

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