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Efficient transformation of potato (*Solanum tuberosum* L.) using a binary vector in *Agrobacterium rhizogenes*

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Summary. We transformed three potato (*Solanum tuberosum* L.) genotypes by using *A. rhizogenes* or a mixture of *A. rhizogenes* and *A. tumefaciens*. Inoculations of potato stem segments were performed with *Agrobacterium rhizogenes* AM8703 containing two independent plasmids: the wild-type Ri-plasmid, pRI1855, and the binary vector plasmid, pBI121. In mixed inoculation experiments, *Agrobacterium rhizogenes* LBA1334 (pRI1855) and *Agrobacterium tumefaciens* AM8706 containing the disarmed Ti-plasmid (pAL4404) and the binary vector plasmid (pBI121) were mixed in a 1:1 ratio. The T-DNA of the binary vector plasmid pBI121 contained two marker genes encoding neomycin phosphotransferase, which confers resistance to kanamycin, and β-glucuronidase. Both transformation procedures gave rise to hairy roots on potato stem segments within 2 weeks. With both procedures it was possible to obtain transformed hairy roots, able to grow on kanamycin and possessing β-glucuronidase activity, without selection pressure. The efficiency of the *A. rhizogenes* AM8703 transformation, however, was much higher than that of the "mixed" transformation. Up to 60% of the hairy roots resulting from the former transformation method were kanamycin resistant and possessed β-glucuronidase activity. There was no correlation between the height of the kanamycin resistance and that of the β-glucuronidase activity in a root clone. Hairy roots obtained from a diploid potato genotype turned out to be diploid in 80% of the cases. Transformed potato plants were recovered from *Agrobacterium rhizogenes* AM8703-induced hairy roots.

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Introduction

Agrobacterium tumefaciens and its Ti-plasmid have been used extensively as vectors to introduce foreign DNA into plants (Fraley et al. 1986). Normally, the plasmids are disarmed by removing the oncogenes responsible for the synthesis of growth hormones and replacing them with selectable or screenable markers. One vector method is the binary vector approach. With this method, a foreign gene is inserted into a disarmed T-DNA plasmid that can replicate in *E. coli* and in *Agrobacterium*. Transfer of the T-DNA to the plant is obtained by a second plasmid in *Agrobacterium* which contains the so-called vir-genes. The former, so-called binary vectors, are available in different forms (Hoekema et al. 1983; Bevan 1984; van den Elzen et al. 1985).

Agrobacterium rhizogenes also has the ability to transfer and integrate T-DNA from a large plasmid, the Ri-plasmid, into the plant genome (Chilton et al. 1982; Willmitzer et al. 1982). In contrast to *A. tumefaciens*, *A. rhizogenes* induces so-called hairy roots instead of tumors, which in some species are capable of regenerating into fertile plants (Tepfer 1984; Ooms et al. 1985a, b; Sukhapinda et al. 1987).

Potato (*Solanum tuberosum* L.) can be transformed and regenerated into plants by using wild-type *Agrobacterium tumefaciens* strains (Ooms et al. 1983), binary vector harboring disarmed *A. tumefaciens* strains (An et al. 1986; Ooms et al. 1987; Sheerman and Bevan 1988; Visser et al. 1989) or wild-type *A. rhizogenes* strains (Ooms

et al. 1985a; Hänisch ten Cate et al. 1988). While transformation with *A. tumefaciens* gives rise to tissue which tends to polypoloidize (Hänisch ten Cate and Sree Ramulu 1987), *A. rhizogenes* induces hairy roots which appear genetically stable (Hänisch ten Cate et al. 1988). Recently, it was shown by Simpson et al. (1986) that a binary vector incorporated in wild-type *A. rhizogenes* (i.e. combining the use of binary vectors with the use of *A. rhizogenes*) gives frequent co-transfer of vector T-DNA and Ri-plasmid T-DNA in tobacco. Here we report on the use of a virulent *A. rhizogenes* strain with an incorporated binary vector to obtain hairy roots on potato, which were analyzed for their non-selected kanamycin resistance and β-glucuronidase activity as well as for their ploidy level. Since transgenic shoots could easily be obtained from these roots, this system provides a general and efficient transformation system for potato without using a selective regime.

Materials and methods

Plant materials

Stem segments without axillary buds were used for transformation experiments. They were obtained from in vitro-grown *Solanum tuberosum* H^2 578 (=PD007, 2n=2x=24), 86.040 (2n=1x=12 and 2n=2x=24) and BD86 (cv Calori, Hettema Zonen BV. Emmeloord, 2n=4x=48).

Plants were grown in jars under 14-h light (3000 lx) at 20°–22 °C on MS medium (Murashige and Skoog 1962), supplemented with 30 g/l sucrose (MS 30).

Bacterial strains and vectors

Bacterial strains and vectors are listed in Table 1.

Binary vector introduction into Agrobacterium

The binary vector pBI121 was constructed by T. Kavanagh (Jefferson et al. 1987). Transfer of pBI121 from *E. coli* MC1022 to *Agrobacterium tumefaciens* LBA4404 and to *Agrobacterium rhizogenes* LBA1334 was by conjugation in the presence of *E. coli* strain HB 101, containing the plasmid pRK2013 to mobilize the vector (see for details Visser et al. 1989). Selection for strains containing the vector was for *A. tumefaciens* on LB (Maniatis et al. 1982) and for *A. rhizogenes* on LC (same composition as LB except 8 g/l NaCl) or MMA medium (Davis and Mingioli 1959). Selection was on rifampecin (100 mg/l) and kanamycin (50 mg/l) and, in the case of *A. rhizogenes*, also spectinomycin (200 mg/l). Integrity of the plasmids in *Agrobacteria* was verified according to Holmes and Quigley 1981).

Inoculum preparation

Inocula of the various *Agrobacteria* strains were prepared by growing the bacteria in liquid LB or LC medium with the indicated antibiotics at 30 °C on a rotary shaker. Overnight cultures of *A. rhizogenes* AM8703 (denoted AM8703) or a 1:1 mixture of *A. rhizogenes* LBA1334 and *A. tumefaciens* AM8706 (denoted LBA1334/AM8706) were used for transformation experiments. In the case of the *A. rhizogenes* AM8703 transformation, the "empty" *A. tumefaciens* LBA4404 was included (also in a 1:1 ratio), to have similar amounts of different bacteria present when inoculating (Fig. 1).

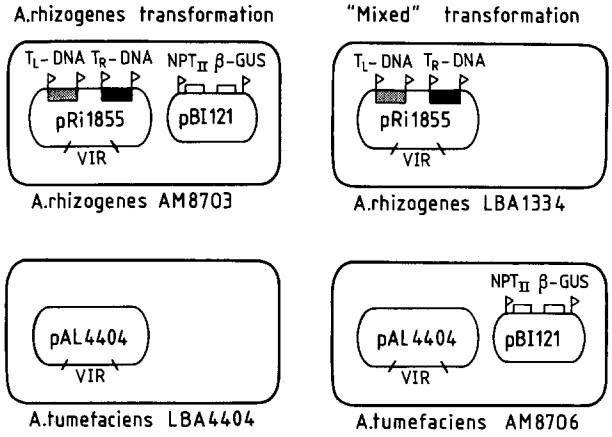

Fig. 1. Schematic drawings of the *Agrobacteria* present in the *A. rhizogenes* and in the "mixed" transformation procedure. Termini sequences are indicated by *flags*. *Black boxes* indicate the TR-DNA which contains auxin and opine sequences. Vir: virulence regions; NPT-II: neomycin phosphotransferase II gene; β-GUS: β-glucuronidase gene

Table 1. Bacterial strains and vectors

	Description	Reference
Plasmids		
pBI121	pBIN19+PCaMV-βGUS-Tnos	Jefferson et al (1987)
pRK2013	IncP, KanR, can mobilize pRK290 and its derivatives	Ditta et al. (1980)
Escherichia coli strains		
HB101	F-, recA, supE44	Boyer and Roulland-Dussoix (1969)
MC1022	803, $supE^+$, $supF^+$	Cassadaban and Cohen (1980)
Agrobacterium strains		
LBA4404	*A. tumefaciens*, avirulent derivative of pAL4404 octopine Ti-plasmid ACH5, RifR	Ooms et al. (1982)
LBA1334	*A. rhizogenes*, pRi1855 plasmid in C58, RifR, SpecR	Offringa et al. (1986)
AM8706	*A. tumefaciens* LBA4404+pBI121, RifR, KanR	This paper
AM8703	*A. rhizogenes* LBA1334+pBI121, RifR, SpecR, KanR	This paper

Inoculations

Stem segments (10 × 2–3 mm) were collected from in vitro-grown plants, immersed in the bacterial suspensions and left there for about 15 min. The stem segments were blotted dry on sterile Whatman 3 MM filter paper and placed on agar-solidified MS 30 plates without antibiotics. The plates were sealed with parafilm. After 2 days, the stem segments were transferred to fresh MS 30 plates supplemented with 200 mg/l cefotaxime (8 stems per plate). The plates were not sealed but instead closed with two pieces of cellophane tape to obtain a good gas exchange and placed at 20 °C, with a 14-h photoperiod. From 10 days on, roots appeared. The roots were excised and transferred to agar-solidified MS 30 medium (hormone free) containing cefotaxime and kanamycin (100 mg/l) or cefotaxime alone. Roots were subcultured at least twice and their length was measured periodically. They were considered kanamycin resistant when they had at least doubled their length in 14 days and were still growing at the end of this period.

Growth assays

Root clones of *S. tuberosum* PD007 transformed with *A. rhizogenes* AM8703 were analyzed for their kanamycin resistance by placing root tips on medium containing kanamycin (0–250 mg/l). After subculturing at least once, increase in length was estimated after 14 days.

β-Glucuronidase assay (GUS-assay)

Two types of GUS-assays were employed: I, a qualitative assay to screen large numbers of samples; II, a quantitative assay to determine the amount of β-GUS activity. For both assays, an extract was made by grinding root material (from one single root up to 100 mg of root material) in 75–150 µl extraction buffer (50 m*M* $NaPO_4$, pH 7.0, 1 m*M* EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10 m*M* beta-mercaptoethanol, Jefferson et al. 1987) with sea sand in an Eppendorf tube. After 1 min centrifugation, the reaction was started by adding 40–50 µl of the supernatant to 4-methyl-umbelliferyl β-D-glucuronide (1 m*M* final concentration), in the case of assay I in microtiter wells, and for assay II in Eppendorf tubes. After 90 min at 37 °C the reaction was terminated with the addition of 150 µl (assay I) or 1.5 ml (assay II) 0.2 *M* Na_2CO_3.

In Assay I, data were obtained by putting the microtiter plates on a long-wave UV illuminator and observing the blue fluorescence. In assay II, the fluorescence was measured with excitation at 365 nm, emission at 455 nm on a Perkin Elmer Spectrofluorimeter type 204, with slit widths set at 3 nm. The fluorimeter was calibrated with a freshly prepared solution of 1 µ*M* 4-methyl-umbelliferone (Mu) in the same buffer. Protein concentrations of root extracts were determined by the Bradford method (1976) with a kit supplied by BIO-RAD Laboratories.

Shoot regeneration

Pieces from hairy root clones were placed on callus induction medium M 433 (agar-solidified MS, supplemented with 20 g/l sucrose, 0.12 mg/l 2.4-D, 2 mg/l zeatin) supplemented with cefotaxime (200 mg/l) and kanamycin (50 mg/l), and transferred to plant regeneration medium M 425 (agar-solidified MS, supplemented with 30 g/l sucrose, 2.25 mg/l BAP, 10 mg/l GA_3) supplemented with cefotaxime (200 mg/l) and kanamycin (50 mg/l), when green calli of about 15 mm^2 had formed. The plates were closed with two pieces of cellophane tape. After 2–3 weeks on M 425, shoots were excised from the calli and transferred to MS 30 plus cefotaxime (200 mg/l). The different cultures (roots, calli and shoots) were maintained under a 14-h photoperiod (3000 lx) at 20 °C. Regenerated shoots were subcultured on MS 30 plus cefotaxime (200 mg/l).

Ploidy level determination

Chromosome numbers of transformed roots were analyzed in mitotic metaphases of root tips according to Pijnacker and Ferwerda (1984).

Opine assays

The presence of agropine and mannopine in transformed roots was established by analyzing crude extracts by paper electrophoresis and silver-staining, according to Petit et al. (1983).

Results

Induction and analysis of hairy roots

After transferring the binary vector pBI121 to *A. tumefaciens* and *A. rhizogenes*, stem segments were inoculated with various bacterial suspensions. Within several days after infection by either combination of bacteria, a visible swelling of the ends of the stem segments could be seen and within 2 weeks, numerous roots developed. Stem segments which did not form roots succumbed. Table 2 gives the number of treated stem segments and the number of root-forming segments. Both rooted and non-rooted stem segments were susceptible to overgrowth by *Agrobacteria*.

In general it seemed that segments of the homozygous diploid *S. tuberosum* 86.040 were more susceptible to overgrowth by *Agrobacteria* and death than were those of the tetraploid *S. tuberosum* BD86. No differences could be seen in root formation per stem segment between the three different sets of bacterial suspensions. Each wound site produced more than one hairy root; we generally selected one root per wound site, each selected root thus representing an independent transformant. As can be seen from Table 2, 75%–86% of the inoculated stem segments gave rise to root formation, depending on

Table 2. Comparison of hairy root induction on potato stem segments inoculated with various *Agrobacteria* strains with or without the binary vector pBI121.

Potato genotype	Bacterial strains	Total no. of inoculated stem segments	No. of stem segments with roots (%)
86.040	LBA1334/AM8706	149	118 (79%)
BD86	LBA1334/AM8706	120	101 (84%)
86.040	AM8703[a]	153	115 (75%)
BD86	AM8703[a]	180	155 (86%)
86.040	LBA1334	664	530 (80%)
BD86	LBA1334	20	15 (75%)

[a] *A. tumefaciens* LBA4404 included as described in "Materials and methods"

Table 3. Kanamycin resistance and β-glucuronidase activity distribution in excised roots of transformed potato. Km = kanamycin

Potato genotype	Bacterial strains	Total no. of excised roots	% Roots growing[a] on MS30			No. of roots tested for β-GUS activity (positive)[b]
			without Km	100 mg/l Km selection		
				1st	2nd	
86.040	LBA1334/AM8706	223	57	13	0	127 (0%)
BD86	LBA1334/AM8706	291	63	27	3	139 (3%)
86.040	AM8703[c]	114	67	47	46	14 (93%)
BD86	AM8703[c]	211	79	61	49	15 (100%)
86.040	LBA1334	45	71	0	0	10 (0%)

[a] Growth is defined as: at least doubling of the length of the root and still growing after 14 days
[b] Beta-glucuronidase activity measured as described in "Materials and methods". Because of the relatively low amount of kanamycin-positive hairy roots in the case of the mixed transformation LBA1334/AM8706, roots which grew on medium without kanamycin were tested. In the AM8703 transformation only roots growing on kanamycin containing medium were tested
[c] *A. tumefaciens* LBA4404 included as described in "Materials and methods"

the genotype used, showing that both the *A. rhizogenes* AM8703 and the "mixed" transformation system are very efficient. Control experiments performed with *A. rhizogenes* LBA1334 alone show that the introduction of the binary vector pBI121 or the presence of *A. tumefaciens* does not influence the efficiency of root formation on stem segments of either potato genotype. Transferring infected stem segments directly to selective medium resulted in a dramatic increase of dead stem segments and also in a reduction of the number of roots per stem segment (data not shown).

In Table 3, the results of the growth of excised roots on kanamycin and the GUS-test are shown. *A. rhizogenes* AM8703 transformation produced 46%–49% hairy roots that are kanamycin resistant, whereas the "mixed" transformation produced only 0%–3%. With one exception, roots which were considered kanamycin resistant also expressed GUS-activity. Roots obtained from wild-type (LBA 1334) *A. rhizogenes*-transformed stem segments were unable to grow on MS 30 medium with 100 mg/l kanamycin and they never showed detectable GUS activity (Table 3).

Experiments in which the LBA1334/AM8706 ratio was decreased from 1:1 to 1:25 indicate that the efficiency of the mixed transformation can be improved. The number of hairy roots producing stem segments in these experiments was much lower, but still adequate (data not shown).

The high percentage of doubly transformed roots obtained by the *A. rhizogenes* AM8703 transformation must be the result of simultaneous integration of the Ri-plasmid T-DNA and the vector T-DNA. The total number of Ri-transformed roots (as judged by growth on hormone-free MS 30 medium) is in the same order of magnitude for both types of transformations: approximately 60%. The potato genotype BD86 seems to react better on the transformation experiments in general. In all experiments, the transformed roots displayed the typical hairy root phenotype, although roots from *S. tuberosum* BD86 showed a more extensive lateral branching than those of the genotype 86.040. With both potato genotypes and with either transformation procedure, a considerable percentage of roots grows on kanamycin after the first selection. In the case of the "mixed" transformation procedure this percentage is almost completely reduced to zero when these roots are transferred for a second time to fresh kanamycin-containing medium. This loss of resistance is not so drastic with roots obtained from the *A. rhizogenes* AM8703 transformation.

Table 4. Ploidy levels of hairy roots induced by *A. rhizogenes* AM8703 in potato

Genotype	No. of roots analyzed	Distribution of various ploidy levels/types			
		x	2x	4x	mixoploid
86.040 (2n = x = 12)	10	1	6	3	0
86.040 (2n = 2x = 24)	20	0	16	3	1
H^2578 (2n = 2x = 24)	15	0	15	0	0
BD86 (2n = 4x = 48)	11	0	0	11	0

Table 4 shows that for a large proportion of the roots, the ploidy level is identical to the ploidy level of the stem segments on which the roots were induced. However, roots obtained from a monoploid 86.040 were almost all polyploidized.

Shoot regeneration from hairy roots

A small-scale experiment was set up with the diploid potato PD007 (H^2578). This was done for two reasons: PD007 can easily be regenerated into shoots from callus

Table 5. Characteristics of PD007 ($H^2$578) hairy clones transformed with *Agrobacterium rhizogenes* AM8703. Km = kanamycin

Ri-PD007 root clone	Opine synthesis[a]	β-GUS activity (fluorescence/mg protein)[b]	Maximum Km concentration (mg/l)[c]	No. of shoots regenerated
71	M/A	1,218	100	10
72	M/A	691	50	11
91	M/A	3,664	150	4
96	M/A	13,808	100	7
106	M/A	8,934	50	10
110	M	8,018	100	12
145	M	7,660	50	10
161	M	99,910	50	–[d]
166	M	2,840	50	6
Controls				
PD007 (untransformed)		0	0	
PD007 (transformed) with *A. rhizogenes* LBA1334		180	10–25	

[a] *M*annopine, *A*gropine
[b] Fluorescence in arbitrary units, mean of three experiments
[c] Maximum kanamycin concentration which allows growth comparable to growth on kanamycin-free medium
[d] Callus was formed, but shoots could not be regenerated

(Jacobsen 1986) and it can be used in crosses to study the inheritance of the Ri, kanamycin and β-glucuronidase traits. After transformation of PD007 stem segments with *A. rhizogenes* AM8703, 76 independent root clones were obtained. Thirty-two of these clones (42%) exhibited growth on MS 30 medium supplemented with 50 mg/l kanamycin. Nine root lines were chosen for further experiments. Mannopine and/or agropine synthesis was detectable in all clones (Table 5), indicating that functional Ri TR-DNA must be present in some of them. Three clones (nos. 110, 145 and 166) did not show a detectable level of agropine synthesis. All nine kanamycin-resistant root lines contained β-glucuronidase activity, as judged by the UV-illuminator test. The original root clones were retested after 4 months growth on MS 30 medium supplemented with cefotaxim and kanamycin, for kanamycin resistance and β-glucuronidase activity (Table 5).

Three different classes of root clones could be distinguished with respect to the β-glucuronidase activity: low levels of GUS (root clones 71, 72, 91 and 166), moderate levels (root clones 106, 110 and 145) and high levels (root clones 96 and 161). The maximum kanamycin concentration which still allows growth differs between the root clones, but seems not to be correlated with the level of β-glucuronidase activity. Roots from untransformed PD007 plants were never able to grow on kanamycin-containing medium nor did they possess any β-GUS activity. Hairy roots from PD007 transformed with wild-type *A. rhizogenes* showed some growth on medium containing maximally 25 mg/l kanamycin; furthermore, these roots did contain a very low β-GUS activity, apparently.

The nine root clones, which were all diploid, were transferred to callus induction medium (M 433) supplemented with kanamycin. After 2 weeks, green calli were transferred to plant regeneration medium (M 425) with kanamycin. Shoots appeared after 2–3 weeks, they were then cut off and subcultured on MS 30 medium with cefotaxim. From each root line a number of shoots was regenerated (Table 5). Only one root line was unable to regenerate shoots.

Discussion

We have used the binary vector pBI121 (Jefferson et al. 1987) in *Agrobacterium rhizogenes* AM8703 and in *A. tumefaciens* AM8706, the latter in combination with *A. rhizogenes* LBA1334, in transformation experiments aimed at obtaining kanamycin-resistant hairy roots possessing β-glucuronidase activity.

The efficiency of root formation on stem segments, using either inoculum, is comparable with that obtained by other investigators in alfalfa, soybean and tobacco (Simpson et al. 1986; Sukhapinda et al. 1987). In terms of efficiency of obtaining kanamycin-resistant hairy roots, *A. rhizogenes* AM8703 is much more effective than the mixture of *A. rhizogenes* LBA1334 and *A. tumefaciens* AM8706. However, Petit et al. (1986) showed that with mixed inoculations, a high percentage of doubly transformed roots can also be obtained. In our hands, a higher percentage of doubly transformed roots was obtained when the ratio *A. rhizogenes*: *A. tumefaciens* was changed from 1:1 to 1:25. As the efficiency of transformation might differ according to the *Agrobacterium* strain used, it could be worthwhile to test other *Agrobacterium* strains in mixed inoculation experiments.

As can be seen from Table 3, there is a considerable escape in the selection of kanamycin-resistant hairy roots. This phenomenon occurred in all three potato genotypes used in this study. The reason for this is not clear, but the observation that LBA1334-transformed roots are sometimes able to grow on low kanamycin concentrations (25 mg/l) might be an explanation. This implies, however, that in transformation experiments all roots should at least be retested once for their resistance. However, the most likely explanation for the observed phenomenon would be chimaerism, leading to loss of kanamycin resistance as the roots grow.

The transformation efficiency obtained with the binary vector is much higher when it is integrated in *A. rhizogenes* than when it is integrated in *A. tumefaciens*. Up to 60% transformation is obtained with *A. rhizogenes*

(Table 3), whereas with *A. tumefaciens* the maximum percentage ranges from 9% (Visser et al. 1989) to 20% (Sheerman and Bevan 1988). With respect to kanamycin resistance, we observed a very large difference between *A. tumefaciens* – and *A. rhizogenes*-transformed material, both in the level of resistance, which was much higher after Ri-transformation, and in the stability of expression. Whereas in *A. rhizogenes*-transformed material, the resistance was unaltered even after prolonged culturing of roots on kanamycin-free medium, we observed a decrease in resistance with *A. tumefaciens*-transformed material (Visser et al. 1989).

In most cases, roots which were kanamycin resistant also showed β-glucuronidase activity. Only 1 root out of 29 kanamycin-resistant roots was not β-glucuronidase positive. However, the results presented in Table 5 show that the root clones behave differently with respect to the β-glucuronidase activity and the maximum kanamycin concentration at wich they still grow. No correlation was found between the level of expression of these two T-DNA traits.

The stability of the ploidy level (Table 4) is very good, especially for roots obtained from diploid and tetraploid plants. Approximately 80% of the hairy roots obtained from diploid stem segments are diploid. Roots from monoploid plant material are almost all polyploidized, which was also reported for other monoploid-derived hairy roots (de Vries-Uijtewaal et al. 1988).

The ability to easily regenerate plants from hairy roots is very important in trying to use this transformation procedure on a broader scale. By using *A. rhizogenes* AM8703 to transform the potato genotype PD007, we were able to obtain diploid hairy root clones from which shoots could be regenerated. The regeneration percentages vary in PD007 from 35% to 54% (Visser 1989).

Plants from nine of these root clones were selected for further analysis. The regenerated shoots will be analyzed genetically to see whether the three introduced traits – Ri-phenotype, kanamycin resistance and β-glucuronidase activity – can be transferred in a stable fashion both vegetatively and generatively.

In conclusion, we can state that it is evident that *A. rhizogenes* transformation is superior to *A. tumefaciens* transformation for several reasons: (i) The transformation efficiency is much higher with *A. rhizogenes* than with *A. tumefaciens*. (ii) The high efficiency allows the screening of hairy roots for a particular trait of interest, without using the kanamycin resistance marker for selection, thus offering the possibility to use constructs without this selection marker. (iii) Within as little as 6 weeks, plants can be obtained. (iiii) In contrast to *A. tumefaciens* – transformed tissue, hairy roots, except for those derived from monoploid plant material, are much more stable with respect to their ploidy level (Hänisch ten Cate et al. 1988; de Vries-Uijtewaal et al. 1988; this report).

The results obtained with different (homozygous) diploid and tetraploid potato genotypes suggest that the binary vector-T-DNA integrated in *A. rhizogenes* could become an alternative transformation system for potato, at least in those cases where initial presence of Ri T-DNA is of no concern.

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