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Expression and inheritance of inserted markers in binary vector carrying *Agrobacterium rhizogenes*-transformed potato (*Solanum tuberosum* L.)

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Summary. Transgenic shoots were regenerated from eight diploid potato hairy root clones obtained by transformation with *Agrobacterium rhizogenes* harboring next to its wild-type Ri-plasmid a binary vector containing the neomycin phosphotransferase and the β-glucuronidase genes. The plants exhibited the typical hairy root phenotype. Of the plants isolated, 58% were tetraploid and 38% were diploid. Flowering and tuberization was much better in the diploid than in the tetraploid plants. Transgenic plants formed a significantly larger root system when grown on kanamycin-containing medium as compared to growth on kanamycin-free medium. Direct evidence for genetic transformation was obtained by opine, neomycin phosphotransferase and β-glucuronidase assays, and by molecular hybridization. Fourteen flowering diploid plants were reciprocally crossed with untransformed *S. tuberosum* plants, but only six were successful. Seedlings obtained from four crosses showed that all traits were transmitted to the offspring. Molecular analysis confirmed the presence of multiple integrations (copies) of both vector T-DNA and Ri-T-DNA. The genetic data, furthermore, suggest that the traits derived from Ri-T-DNA and binary vector T-DNA are linked, as no recombination between the different traits was observed.

Key words: *Agrobacterium rhizogenes* – *Solanum tuberosum* – Binary vector – Neomycin phosphotransferase – β-Glucuronidase

Introduction

Binary and co-integrate vectors introduced in virulent *A. rhizogenes* strains have been used to transform a number of plant species. Frequent co-transformation of Ri-T-DNA and vector T-DNA could be demonstrated in all cases (Simpson et al. 1986; Sukhapinda et al. 1987a, b; Morgan et al. 1987; Visser et al. 1989b). Genetic analysis has been restricted to tomato, alfalfa and tobacco, and most attention has been directed to the stability and inheritance of the integrated vector T-DNA instead of to the segregation of both Ri-T-DNA and vector T-DNA in the progeny (Shahin et al. 1986; Jouanin et al. 1987; Sukhapinda et al. 1987a, b).

In the few reports on the segregation of different T-DNAs it was shown, however, that simultaneous co-transformation of these T-DNAs can result in the insertion on different genetic loci. Most of these data were derived from studies on the TL- and TR-T-DNAs from *A. rhizogenes* (Taylor et al. 1985; De Framond et al. 1986; Peerbolte 1986). Data derived from transformations with different *Agrobacteria* strains with different T-DNAs gave similar results (Petit et al. 1986; McKnight et al. 1987). Because of the possibility of recombination between loci during meiosis, this could make the wild-type *A. rhizogenes* a suitable vehicle to introduce foreign genes into crop plants.

Previously it was shown that potato hairy root clones, showing characters brought about by both Ri-T-DNA and binary vector T-DNA, could be obtained efficiently under non-selective conditions (Visser et al. 1989b).

Here we report on the biochemical and molecular characterization of potato plants derived from such hairy root clones. These regenerated plants contain and express Ri-T-DNA and vector T-DNA and transmit the traits encoded by these DNAs stably during shoot and

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tuber propagation. Furthermore, the characterization of diploid Ri-transformed plants used in crosses with untransformed diploid potato plants and the analysis of progeny plants is described.

Materials and methods

Plant materials

Shoots were grown in vitro on kanamycin-free or kanamycin-containing (50 mg/l) MS 30 (MS, Murashige and Skoog 1962) medium supplemented with 200 mg/l cefotaxim. These shoots had been obtained from diploid kanamycin-resistant, β-glucuronidase positive hairy root clones (Ri-71, 72, 91, 96, 106, 110, 145 and 166) obtained after transformation of stem segments of the diploid *Solanum tuberosum* PD007 (= H^2578) with *Agrobacterium rhizogenes* AM8703, carrying the wild-type Ri-plasmid, pRI1855, and the T-DNA vector, pBI121 (Visser et al. 1989b).

In vitro-grown shoots were transferred to soil in a greenhouse (16-h photoperiod, 18 °C day, 16 °C night).

In vitro tuber induction was performed essentially as described by Hovenkamp-Hermelink et al. (1988), but when subculturing axillary shoot segments of transgenic plants, the tuber induction medium (M 388) was supplemented with 200 mg/l cefotaxim and 50 mg/l kanamycin.

Diploid potato clones 870008 (Sta), 870007 (Stb), 871026-4 (Stc) and 871016-1 (Std) were used for crossing. Plants were maintained in the greenhouse.

DNA isolation and Southern blot analysis

Total DNA was isolated from 500 mg leaf material according to Dellaporta et al. (1983). EcoRI and HindIII restriction enzymes were used to digest the DNA. Digests were analyzed using the Southern blot technique (Southern 1975). DNA was separated by agarose gel electrophoresis and blotted onto Gene Screen Plus membranes (Maniatis et al. 1982; Visser et al. 1988, 1989a). Plasmid DNA isolation was according to Birnboim and Doly (1979). Hybridizations were performed for 40 h at 65 °C with clones pMP27 and pMP66 (respectively, TR and TL area of the T-DNA of *Agrobacterium rhizogenes* pRI 1855, Pomponi et al. 1983), and with a clone encompassing the NPT-II gene. Probes were made radioactive using the random primer labelling technique (Feinberg and Vogelstein 1983).

Genetic analysis of transformed plants

Diploid Ri-007 plants which flowered well were reciprocally crossed with untransformed *S. tuberosum* plants. Resulting seeds were germinated after sterilization. Seeds were sterilized by a 30-s wash in 96% ethanol, a 5-min wash in saturated calcium hypochloride with 0.1% SDS, followed by three washings in sterile aqua bidest. The seeds were placed on filter paper moistened with sterile water containing 10 ppm GA_3. After 3 days, part of the seed coat opposite to the embryo was cut off to enable the embryo to develop more quickly. After cutting, the seeds were placed on MS 30 medium in a growth chamber under 14-h light (3000 lx) at 20°–22 °C.

After germination, the primary root of each seedling was cut off and used for the β-glucuronidase (β-GUS) assay, while the remainder of the seedling was transferred to MS 30 medium containing 50 mg/l kanamycin. The plates were incubated under the same conditions as described above. The root formation was recorded after 8 days. Material from seedlings resulting from crosses in which one of the parents was agropine positive was analyzed for the presence of this opine, by paper electrophoresis and silver staining of crude extracts, according to Petit et al. (1983).

Progenies were screened for the occurrence of Ri-phenotype – plants forming roots which showed excessive branching and lacked geotropism and/or compound wrinkled leaves.

Miscellaneous

Cytological analysis, growth assays on kanamycin-containing medium, β-glucuronidase (GUS), NPT-II and opine assays were performed, as described previously (Visser et al. 1988, 1989b). Adventitious shoot regeneration on stem and leaf segments of Ri-007 transformants was as described by Hovenkamp-Hermelink et al. (1988) with some modifications. After floating on M 387 medium overnight, the segments were transferred to M 425 medium (MS supplemented with 2.25 mg/l BAP, 10 mg/l GA_3 and 30 g/l sucrose) for shoot regeneration.

Pollen stainability was determined as the percentage of pollen stainable with orceine. Pollen was also stained with Lugol's solution (I-KI; Merck) to determine the amount of starch present. Pollen which did not react with Lugol (thus giving a yellow colour) or pollen which stained dark blue with Lugol was counted. Pollen which was visibly damaged was discarded. The percentage of yellow colouring pollen was taken as a measure for the viability of the pollen.

Results

Phenotypical characterization of in vitro-grown shoots

Sixty-nine shoots were derived from induced green calli, on regeneration medium containing kanamycin (50 mg/l). All root clones, except Ri-91, gave numerous shoots within 6–10 weeks, but only the first-appearing shoot on each callus was selected for further analysis. All selected shoots showed to some degree wrinkled and sometimes compound leaves, abundant root formation and rooting from leaf axils. One root clone (Ri-110) gave rise predominantly to very short shoots with small, dark green, wrinkled leaves looking like miniature "curly kale" (Fig. 1). All shoots rooted in the presence of kanamycin and they all showed GUS-activity, both in extracts of roots and of leaves. Tuberization in vitro on kanamycin-containing medium occurred with 52 shoots. Variation in tuber form, size and eye setting was large for plants derived from different root clones, but relatively small for plants from one and the same root clone. The normal form of in vitro-grown PD007 tubers, oblong shape and superficially set eyes, was only found in regenerants of Ri-96. Most of the in vitro-induced tubers had numerous, deeply set eyes and often formed many roots during the tuberization process.

Phenotypical characterization of soil-grown plants

After transfer to soil, transformed plants additionally showed morphological variations which were not visible during in vitro growth. Variation in height and degree of senescence was visible after 6 weeks. Variation was also

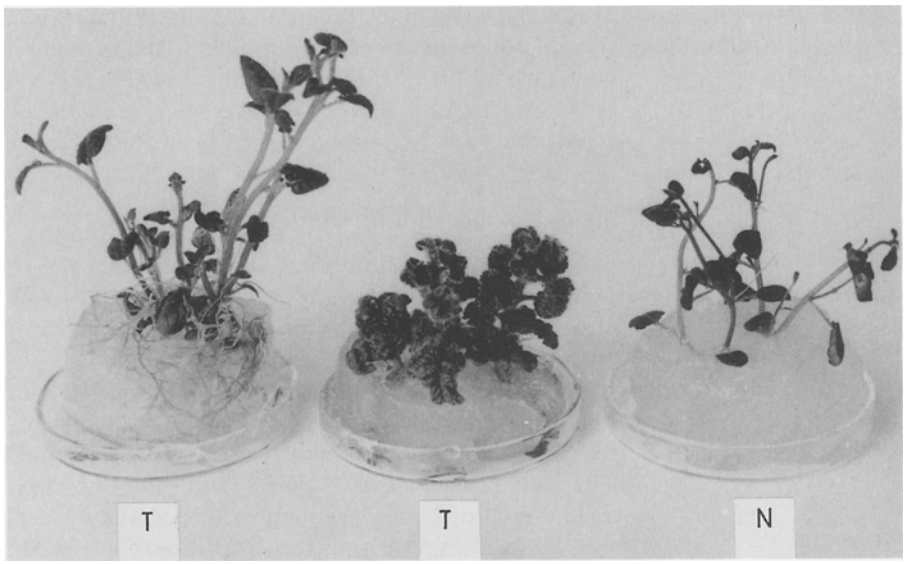

Fig. 1. The morphology of transformed (T) and untransformed (N) potato shoots. In the middle, the "curly kale-like" transformant

Table 1. Ploidy level, ability to flower and tuberization of Ri-007 plants

Plants	Flowering	Diploid		Tetraploid		Other[a]	
		+	−	+	−	+	−
71/1-10		4 (3)[b]	2 (1)	−	4 (1)	−	−
72/1-11		−	−	−	9 (0)	−	2 (0)
91/1-4		1 (1)	2 (2)	−	1 (0)	−	−
96/1-6		−	−	1 (1)	5 (4)	−	−
106/1-10		1 (1)	1 (1)	−	8 (8)	−	−
110/1-12		5 (2)	2 (1)	−	3 (1)	−	2 (1)
145/1-10		2 (1)	1 (1)	1 (1)	5 (0)	−	1 (0)
166/1-6		1 (1)	1 (1)	−	3 (3)	1 (1)	−
Total no. of flowering plants		14 (9)	9 (7)	2 (2)	38 (17)	1 (1)	5 (1)

[a] Aneuploids and mixoploids
[b] Between brackets are given the number of plants showing tuberization in soil

observed for flowering ability and tuberization. All Ri-007 plants showed senescence much earlier than untransformed PD007. All root clones, except clone 72, produced one or more flowering plants (Table 1). Seventeen flowering plants were obtained. Some of the transformants showed a shorter perianth and no full opening of the flowers. All flowers contained pollen stainable with orceine. Only 37 plants formed tubers in soil, none of them from root clone 72 (Table 1). No differences were observed between tubers from transformed and untransformed plants.

Cytological analysis of transformants

The chromosome number of all 69 regenerated shoots was determined. Twenty-three shoots (33%) were diploid ($2n=2\times=24$), 40 were tetraploid ($2n=4\times=48$). Three aneuploids ($2n=4\times=47$) and three mixoploids (24 and 48 chromosomes) were found (Table 1). There exists a strong relationship between flowering and ploidy level: about 61% of the diploid plants flowered, whereas only 5% of the tetraploid plants did. Such a tendency, albeit less clear, can also be seen for tuberization in soil: 70% of the diploid plants and only 46% of the tetraploid plants gave tubers.

Biochemical characterization of regenerants

From each root clone one plant, as far as possible a diploid one, and the corresponding in vitro culture were selected for further biochemical analysis. The roots and leaves of all plants contained mannopine, whereas agropine was present in only four of the five plants which were regenerated from agropine-positive hairy root clones (Fig. 2A). Although shoot 71/1 did not contain agropine, other shoots (71/6 and 71/7) regenerated from this root clone did.

In all in vitro-grown shoots and in all soil-grown plants, NPT-II activity was detectable (Fig. 2B and data not shown). All plants were resistant to kanamycin, but not all the plants contained equal amounts of NPT-II as judged by the ability to inactivate kanamycin in vitro. As can be seen from Fig. 2B, there must be a difference in the amount of NPT-II activity among the different plants. The amount of roots formed on kanamycin-containing medium (50 mg/l) was larger and the roots were also much thicker than on kanamycin-free medium (Table 2). Ri-71/1 shoots displayed yet another feature when they were placed on kanamycin: they sometimes failed to form roots and merely formed callus at the base of the stem.

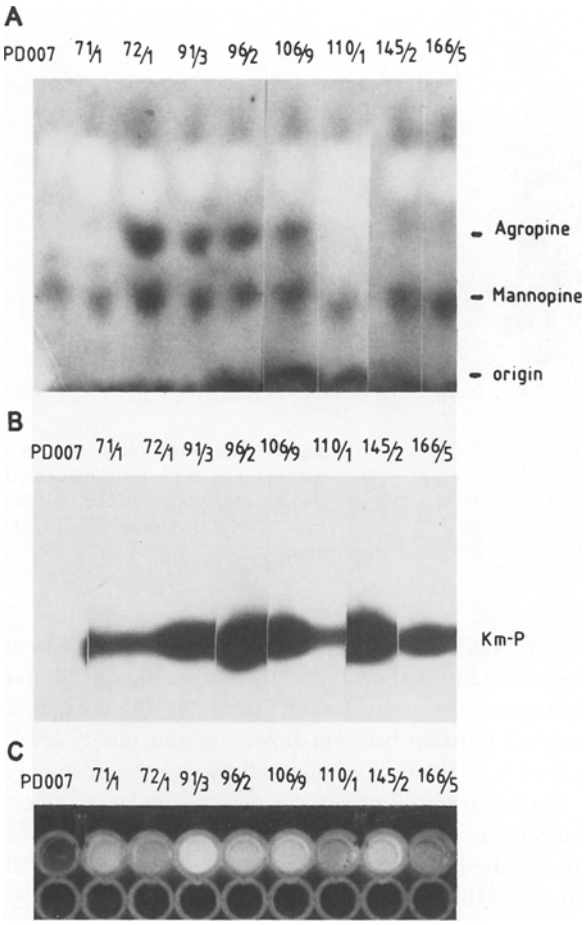

Fig. 2A–C. Biochemical characteristics of Ri-007 transformants. PD007 is an untransformed control. **A** Agropine detection in leaf tissue. **B** Neomycin phosphotransferase enzyme test: KM-P = kanamycin-phosphate. **C** β-Glucuronidase activity in roots (UV-illuminator fluorescence assay)

Table 2. Rooting ability of Ri-007 plants on kanamycin-containing medium

Plant clone	Root weight per shoot after 14 days (mean of six plants)		
	0 mg/l kanamycin	50 mg/l kanamycin	Km-R-factor[a]
71/1	ND[b]	ND	ND
72/1	65 mg	310 mg	4.8
91/3	110 mg	300 mg	2.7
96/2	80 mg	225 mg	2.8
106/9	45 mg	180 mg	4.0
110/1	7 mg	30 mg	4.3
145/2	60 mg	165 mg	2.8
166/5	45 mg	215 mg	4.8
PD007	7 mg	0 mg	ND

[a] Km-R-factor is the ratio of root weight of shoots on kanamycin-containing medium over that on kanamycin-free medium
[b] ND: Not Determined, in the case of clone 71/1 due to callus formation at the base of the stems

GUS-activity in the shoots and in root cultures from these shoots were comparable to those found in the root clones from which the shoots were derived (Fig. 2B and data not shown). GUS-activity was present in leaves, stems, roots and tubers of all tested plants (not shown).

Seed set in crosses with Ri-transformed plants

Pollen stainability with orceine, an indirect reference to fertility, varied from 68% to 89% in the 14 flowering diploid plants. In pollen preparations from a number of transgenic plants, starch granule accumulation was observed when it was stained with Lugol. Starch was not found in fertile pollen from PD007 and other non-transformed clones. Several reciprocal crosses were performed with the diploid Ri-007 plants and untransformed diploid potato clones. The number of pollinations performed per transgenic genotype varied from 5 to 100. Six plants originating from three different root clones (71/1, 71/6, 71/7, 145/2, 145/4 and 166/5) gave seed set. The number of pollinated flowers per berry varied from 3 (for plant 71/1 as female) to 100 (for plant 145/2 as male). The number of seeds per berry varied from 30 to 260.

Molecular characterization: Ri-T-DNA analysis

The DNA from eight Ri-plants (from each root clone one plant) was analyzed, using plasmids pMP66 and pMP27 as probes for the TL and TR area of the pRI 1855 T-DNA, respectively. As shown in Fig. 3A, the shoot clones gave hybridizing bands resulting from the TL-DNA of pRI 1855, which co-migrated with EcoRI fragments 15, 36, 37a and 40 (Fig. 3C), indicating that the internal structure of the TL-DNA as determined by Pomponi et al. (1983) is maintained in all plants. The eight plants contained, in addition, one or several bands of higher molecular weight. Only two plants contained a band of similar molecular weight as fragment 3a (Fig. 3C). Since no evidence for the presence of fragment 34 was found in the plants, extra bands appearing must be border fragments of the left end of the TL region, as the right end of the TL region of pRI 1855 is not included in pMP 66 (Fig. 3C). Therefore, additional bands found in the autoradiogram must result from junction fragments containing part of plasmid sequences joined to plant DNA. The number of these fragments can give a rough estimate of the copy number of TL-DNA in the transformed plants. The number of TL-DNA copies was assessed to be in the region of 1–4. This number is in agreement with reconstructions of copy number determinations of pMP 66 (data not shown).

The presence of agropine in four plants pointed strongly to the presence of TR-DNA. The results in Fig. 3B indicated that only those plants that synthesized agropine (Fig. 2A) contained TR-DNA sequences. In

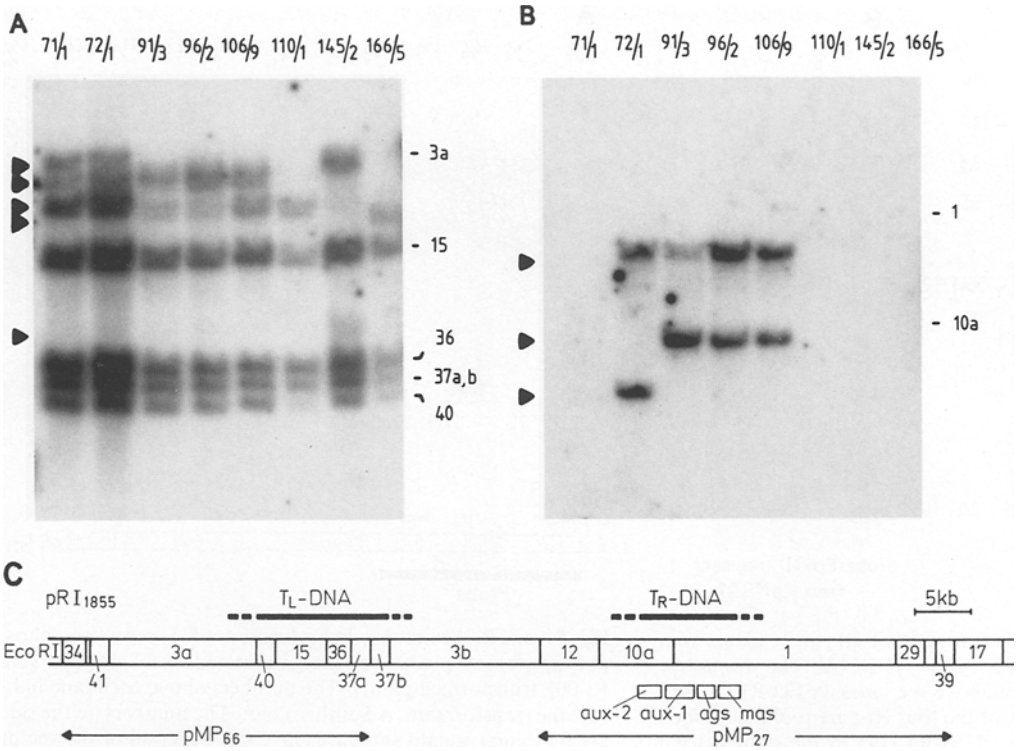

Fig. 3A–C. Molecular hybridization of probes representing the TL- and TR-DNA to EcoRI-digested total DNA from transformed plants. The respective transformants are indicated above each lane. The numbers on the side if the Southern blots refer to the EcoRI fragments on the TL-DNA and the TR-DNA of pRI1855, respectively. *Arrow heads* refer to fragments not found as such in pRI1855 DNA. **A** probed with pMP66 (TL-DNA). **B** probed with pMP27 (TR-DNA). **C** EcoRI restriction map of the T-DNA region of pRI1855. The positions of pMP66 and PMP27, as well as from the auxin 1, auxin 2 and agropine synthase (ags) genes are indicated

the four positive clones, only two fragments could be found, neither of which co-migrated with fragment 1 or 10a (containing the Ags and auxin 1 and 2 genes). This indicated that these two fragments must be border junctions between TR-DNA and plant DNA, which means that there are two TR-DNA copies present in these plants. The intensity of the bands in the Southerns supported this assumption. No hybridization of labelled pMP66 or pMP27 to untransformed plant tissue was observed under the conditions used here (data not shown).

Four Ri-parent plants (71/1, 71/6, 71/7 and 145/4) from which progeny had been obtained were probed with parts of plasmids pMP66 and pMP27, to establish more precisely the number of TL- and TR-DNA copies (Fig. 4A and B). HindIII-restricted DNA from these four plants probed with fragment 3a from pMP66 showed a number of bands, two of which could be identified as fragments H23 and H45; the other bands represent the different junction fragments between TL-DNA and plant DNA. The number of extra bands represents the number of copies, which is in agreement with the data from the EcoRI restrictions. The number of TL-DNA integrations in plant 71/1 is at least three. Plants 71/6, 71/7 and 145/4 contain no more than two TL-DNA copies (Fig. 4A).

TR-DNA sequences were detected in plants 71/6 and 71/7, but not in 71/1 and 145/4. EcoRI- and HindIII-restricted DNA, probed with fragment 1 from pMP27 containing the agropine synthase gene, showed two bands. In EcoRI-digested DNA, the molecular weight of the bands differed from that of fragment 1 (Fig. 5B). The two bands indicated that two TR-DNA copies are integrated in plants 71/6 and 71/7.

Vector T-DNA analysis

In order to establish the presence of vector T-DNA in the transformants, we hybridized EcoRI-digested plant DNA with a probe encompassing the NPT-II gene (Fig. 5B). With EcoRI-restricted DNA, one fragment with a minimum size of ~4.5 kb should be found if the T-DNA were intact. As is shown in Fig. 5A, all plants contained one or more copies of intact T-DNA. Plant 96/2 contained, in addition, a band smaller than 4.5 kb, which must therefore be a truncated copy. Digestion of DNA with HindIII gave, with the exception of clone

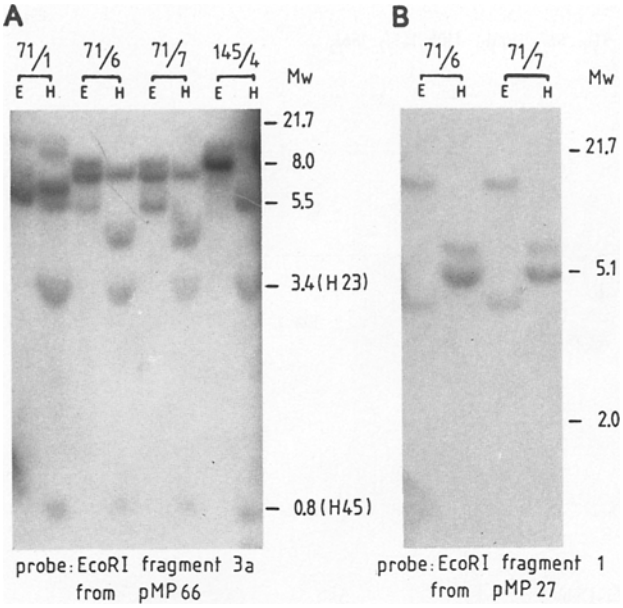

Fig. 4 A and B. Molecular hybridization of Ri-parent plants with probes representing the TL- and TR-DNA. The respective transformants are indicated above the lanes. **A** EcoRI-(E) and HindIII-(H) digested DNA of the four Ri-parent plants probed with a TL-DNA fragment. H23 and H45 in the right margin refer to these respective fragments on HindIII- restricted TL-DNA of pRI1855. **B** EcoRI-(E) and HindIII-(H) restricted DNA of the agropine-positive plants probed with a TR-DNA fragment. Molecular weights are indicated in kb

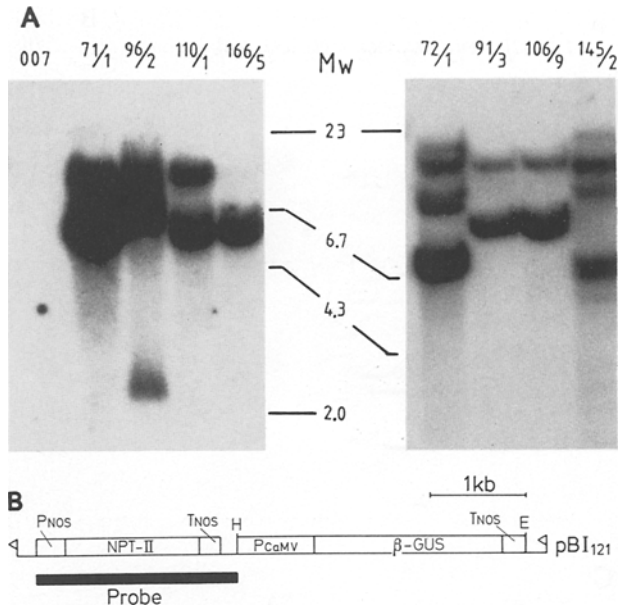

Fig. 5 A and B. Molecular hybridization of a neomycin phosphotransferase probe to EcoRI-digested total DNA from Ri-007-transformed plants. The numbers above each lane indicate the transformant. **A** Southern blot. The numbers on the side are molecular weight standards in kb. **B** Diagram of the vector T-DNA showing the relevant restriction sites and the homologous region between the vector T-DNA and the probe. Flags indicate the T-DNA borders; E, EcoRI; H, HindIII; P_{NOS}, nopaline synthase promoter; NPT-II, neomycin phosphotransferase gene; T_{NOS}, nopaline synthase terminator; P_{CaMV}, 35S cauliflower mosaic virus promoter; β-GUS, β-glucuronidase gene

110/1, similar results and the intensity of the bands supported these findings for all plants (not shown).

DNA from the four Ri-parent plants, when restricted with HindIII and EcoRI and probed with the NPT-II gene fragment (Fig. 5B), showed bands which were larger than the minimum sizes of ~1.6 kb or ~4.5 kb, indicating that complete copies have been integrated (Fig. 6A and B). For plant 71/1, five bands were present in HindIII-restricted DNA, whereas in EcoRI-restricted DNA at least four bands were visible. For both plants 71/6 and 71/7, two (HindIII) and three (EcoRI) bands were found. Plant 145/4 showed five HindIII and five EcoRI DNA bands.

Stability of the introduced traits in tuber-propagated plants

In order to establish whether the introduced marker genes are stably integrated into the genome and transmitted after multiplication, tubers from plant clones 71/1, 106/9, 145/2 and 166/5 were allowed to sprout in vitro on kanamycin-containing (50 mg/l) MS 30 medium. All shoots obtained from these tubers were kanamycin resistant and possessed β-GUS activity like the mother plants. In addition, plant 106/9 synthesized agropine.

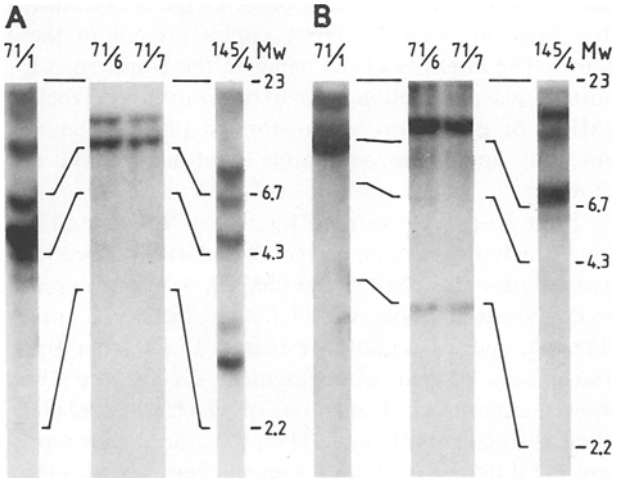

Fig. 6 A and B. Molecular hybridization of the Ri-parent plants with an NPT-II gene fragment. The respective transformants are indicated above the lanes. Molecular weights are indicated in kb. **A** HindIII-digested DNA. **B** EcoRI-restricted DNA

Table 3. Segregation of the transformed traits in progeny of crosses between diploid *S. tuberosum* lines (St) and Ri-007 diploid plants

Family	8801	8802	8803	8804
crosses[a] ♀ × ♂	71/1 × Sta	71/6 × Stb	Stc × 71/7	Std × 145/4
Sown seeds	25	20	20	24
Germinated seeds	24	19	15	22
Germination rate	96%	95%	75%	92%
Phenotype[b]				
(1) TR+, TL+, TV+	–	13	14 (12)[d]	–
(2) TR+, TL−, TV+	–	–	–	–
(3) TR+, TL+, TV−	–	–	–	–
(4) TR+, TL−, TV−	–	–	–	–
(5) TR−, TL+, TV+	22	–	–	2
(6) TR−, TL+, TV−	–	–	–	–
(7) TR−, TL−, TV+	–	–	–	10
(8) TR−, TL−, TV−	2	6	1 (3)	10
Chi-square test[b]:				
1 integration 1:1	18.18 S	2.58 NS	11.26 S	0.19 NS
2 integrations[c] 3:1	3.56 NS	0.44 NS	1.34 NS	7.30 S

[a] Sta (870008), Stb(870007), Stc (871026-4), Std (871016-1)
[b] Phenotype: TR+, agropine positive; TL+, RI-phenotype; TV+, kanamycin resistant and β-glucuronidase positive. TR−, agropine negative; TL−, no Ri-phenotype; TV−, kanamycin sensitive and β-glucuronidase negative. In the case of plants 71/1 and 145/4, no TR-DNA was integrated so only phenotypical classes 5–8 would have been expected
[c] S: statistically significant at 0.05 level; NS: statistically non-significant at 0.05 level. In family 8803, the Chi-square is given only for the β-glucuronidase segregation of the vector T-DNA and in family 8804, only for TV+: TV− segregation
[d] Segregation of the kanamycin trait is given in brackets
[e] Segregating independently

Leaf and stem segments of these vegetatively propagated shoots were able to form adventitious shoots on kanamycin-containing medium. No structural changes in DNA hybridization patterns, as compared to the mother plants, were observed in these four shoots (data not shown). These observations indicated that the introduced traits are stably integrated into the genome.

Analysis of progenies

Seeds obtained from four crosses (Table 3) were germinated and the seedlings were analyzed for the introduced traits. The germination rate was 75%–96% and, although the number of tested seedlings is small, the inheritance of the introduced traits together with the molecular analysis should give useful information about the genotype of the parents. The genetic analysis showed that in all families, segregation for hairy root phenotype (TL+/TL–) and for kanamycin resistance and GUS-activity (TV+/TV–) occurred. In families 8801–8803, no recombinants (of TL and TV) were found. Only in family 8804 did recombination occur. Here the recombinant phenotype TL– TV+ was found. However, since the other possible recombinant TL+ TV– was not found and the TL+/TL– segregation was grossly distorted, the occurrence of the TL– TV+ plants must be ascribed to loss of the active TL gene, rather than to recombination.

Families 8802 and 8803 showed segregation for a third character, agropine production, in accordance with the properties of the parents 71/6 and 71/7, respectively. In both families, only the parent types and no recombinants of TR, TL and TV were found (Table 3). In family 8803, only 12 of the 14 GUS-positive plants were kanamycin resistant. However, with respect to this family, it was noticed that the expression of the kanamycin trait in the seedlings was less prominent than in the other crosses. Kanamycin resistance was not as easy to determine, as one could distinguish, besides resistant and sensitive plants, also a group of plants which had a clearly reduced root development on kanamycin-containing medium. Furthermore, kanamycin-resistant seedlings from this cross needed a much longer time to root on kanamycin-containing medium. Therefore, the presence of GUS-activity in this family was used as a criterium for the TV+ phenotype.

Discussion

Biochemical characterization of transgenic plants

Table 2 clearly demonstrates that more roots were formed by the transgenic shoots on medium with kanamycin. However, the relationship between the presence of kanamycin in the medium and the amount of

roots formed is not clear. In shoots of *A. tumefaciens*-transformed potato, the opposite was always observed – reduction of root formation on kanamycin-containing medium (Visser et al. 1989a). The kanamycin resistance was stably integrated, even after prolonged culture under kanamycin-free conditions in the greenhouse, NPT-II enzyme activity was present in about the same amount as after in vitro culturing on kanamycin-containing medium.

All plants showed GUS-activity (Fig. 2C) and during the whole period that the plants were growing, there was no sign that this trait was lost. Here there does not seem to exist a relationship between the level of expression and the vector T-DNA copy number.

Molecular characterization

Molecular analysis of DNA showed that in all transformants, Ri-fragments 15, 36, 37a and 40 plus extra border fragments were present (Fig. 3A). These are TL-DNA sequences which were previously identified as belonging to the core region (represented by fragments 15, 36, 37a and 40) and harboring the so-called *rol* A, B, C and D genes (Pomponi et al. 1983; Filetici et al. 1987). A similar pattern of hybridization for TL-DNA as shown in Fig. 3A was found in transformed potato and alfalfa plants (Ooms et al. 1985; Spano et al. 1987), whereas in Ri-transformed tobacco, fewer fragments, down to only fragment 15, were present (Costantino et al. 1984). The number of insertion events and thus possible TL-DNA copies (1–4) is in agreement with that reported for other plant species like *N. rustica* and alfalfa (Hamill et al. 1987; Spano et al. 1987; Sukhapinda et al. 1987a). The analysis for the presence of TR-DNA showed that only those plants which produced agropine contained TR-DNA sequences; they contained two hybridizing bands (Figs. 3B and 4B). These two bands resulted from two TR-DNA copies, which could only be the case if no auxin gene or only a part of the auxin-1 gene was present, as was reported by Durand-Tardif et al. (1985) and Peerbolte (1986).

As earlier reported by Hamill et al. (1987) and Spano et al. (1987), also in this case it was found that the number of TR-DNA copies is lower than that of TL-DNA sequences.

The vector T-DNA copy number, as determined by Southern analysis, is relatively low: 1–5 (Figs. 5A, 6A and B). The transfer of a single copy of vector T-DNA is not very common with binary vectors in *A. rhizogenes* (Simpson et al. 1986; Hamill et al. 1987), but has been reported for tomato (Sukhapinda et al. 1987b). In the procedure used with tomato, the cause of the low copy numbers of vector T-DNA was sought in the low level of selection pressure. However, Hamill et al. (1987) showed that even under non-selective conditions, as many as 11 copies of vector T-DNA could be found in *Nicotiana rustica* hairy roots. Since in our experiments hairy root lines were obtained under non-selective conditions (Visser et al. 1989b) potato, in its reaction, resembles tomato rather than *Nicotiana*. Given these results, it seems likely that the number of vector T-DNA copies is also determined by the plant species used.

Genetic analysis

According to Tepfer (1984) and Shahin et al. (1986), reduced male fertility is one of the negative characters of hairy root plants. Our results show that potato does not form an exception to this rule. When pollinations were performed, there was no seed set in the large majority of cases, despite the fact that staining of pollen with orceine showed high percentages of apparently viable pollen. In a number of cases this pollen contained starch. According to De Nettancourt and Eriksson (1968) pollen grains of tomato, after irradiation, accumulated starch and were not able to germinate. This was due to a disturbance in the physiological development of the pollen. The presence of starch in pollen preparations of transformants points to a similar developmental block. These results indicate that for transformed plants, staining pollen solely with orceine does not give a good estimation of its viability. Seeds were only obtained from 6 transformants, 3 in which an Ri-plant was used as male and 3 as a female, out of a total of 14 tested transgenic parents.

Seeds from four crosses were germinated and it is clear from the offspring that the introduced traits are transmitted through meiosis (Table 3). No evidence for recombination between the different T-DNAs was obtained, as we found only parent-type phenotypes in families 8801–8803. Family 8804 did show results of recombination between TL and TV, but here the number of normal (TL–) plants was much higher than expected. Similar results have been obtained for TL-DNA in tobacco (Durand-Tardif et al. 1985) and for vector T-DNA in tomato and tobacco (Sukhapinda et al. 1987b; Jouanin et al. 1987). Until full molecular data on the seedlings points to the contrary, it is likely that transcriptional inactivation of the TL-DNA genes is the reason for this phenomenon, as has recently been demonstrated in a number of *Nicotiana* species (Sinkar et al. 1988).

Uncoupling (of expression) between the two vector T-DNA traits has only been observed in two plants from family 8803 (Table 3). In this particular cross, the kanamycin resistance was not as clear as in the other crosses. This discrepancy of tolerance to kanamycin was also found in tobacco (Jouanin et al. 1987). Whether the uncoupling of kanamycin resistance and GUS-activity is only at the level of expression has yet to be investigated. Molecular analysis of plant 145/4 suggested the presence of five different vector T-DNA copies; the segregation

for TV+/TV− (1:1) suggests that they are located near each other on one chromosome or that a number of the integrated copies are not expressed. The molecular data for plants 71/6 and 71/7 suggest two different integration events both for TL- and TV-DNA, which is in accordance with a 3:1 segregation. TR-DNA segregation data in plants 71/6 and 71/7 are also in accordance with the observed number of TR-DNA copies. For plant 71/1 no firm conclusions can be drawn from the genetic data about the number of different integrations of the TL-DNA or the TV-DNA. However, the relatively high number of TV- and TL-DNA copies and the fact that the genetic analysis was significantly different from a 1:1 segregation means that functional TV- and TL-DNAs are located at at least two genetically different sites on the chromosomes of this plant.

The genetic data show no independent segregation of the characters brought about by Ri-T- and vector T-DNA, but instead suggests complete or rather strong linkage. Complete linkage between introduced characters has been reported to occur only incidentally (Jouanin et al. 1987; Peerbolte 1986). In fact, evidence for non-coordinate transfer of different T-DNAs has been obtained in several studies on a number of plant species (Taylor et al. 1985; De Framond et al. 1986; Petit et al. 1986; McKnight et al. 1987; Sukhapinda et al. 1987). However, in these cases, the different T-DNAs were either not in one and the same bacterium (Petit et al. 1986; McKnight et al. 1987) or they concerned the TL- and TR-DNA region of the Ri-T-DNA (Taylor et al. 1985; Costantino et al. 1984; De Framond et al. 1986). Segregation as well as linkage of TL- and TR-DNA has been reported (Taylor et al. 1985; Costantino et al. 1984; Peerbolte 1986). In some cases this segregation was only found on the level of expression and not on the DNA level (Peerbolte 1986). In our experiments segregation could be expected for the three different T-DNAs. In spite of the fact that some inactivation of genes may occur in progeny plants, segregation other than the one in family 8804 should have been found if the different T-DNAs were integrated at different places in the genome. One way in which our results could be explained is that the different T-DNAs have undergone recombination in *A. rhizogenes,* resulting in a recombinant plasmid carrying all T-DNAs. However, plasmid DNA analysis from the *A. rhizogenes* strain AM8703 showed that the binary vector DNA plasmid was still present unaltered. Another possibility could be tandem integration during the transformation process at the same site. A third, less likely, possibility could be independent integration of the traits, but then by chance in such a way that in each chromosome in which a vector T-DNA copy is integrated, a TL- (and TR-) DNA copy is also inserted, at such distances that recombination is not very frequent. The chances for something like that to happen seem very small.

In conclusion, the results of this study suggest that, although the genetic analysis has been performed on a relatively small number of seedlings, in potato the insertion of Ri-T-DNA and vector T-DNA seems to occur at the same genetic loci. This means that wild-type *A. rhizogenes* is less usable as a vector for the introduction of foreign genes into potato breeding clones.

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