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Frequent spontaneous deletions of Ri T-DNA in *Agrobacterium rhizogenes* transformed potato roots and regenerated plants

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Abstract

The presence of T-DNA was examined by Southern blot analysis in 16 regenerated shoot lines derived from 6 *Agrobacterium rhizogenes*-transformed root clones of *Solanum tuberosum* L. cv. Bintje.

TR-DNA, present in regenerated shoot lines from 3 out of 6 root clones was correlated with the presence of opines. One root clone produced opines up to 2.5 years of subculture. However, plant regeneration from and prolonged subculturing of this root clone resulted in loss of opine synthesis, caused by deletion of TR-DNA.

TL-DNA inserted at 1 to 5 independent loci was found in 14 of the 16 shoot lines. Surprisingly, 1 to 2 additional insertions next to similar insertions of TL-DNA were found in shoot lines from the same root clone (named 'sister' shoot lines) in 2 out of 4 root clones. Nevertheless, this did not result in gross phenotypic variation between sister shoot lines. Another root clone regenerated 1 shoot line with an Ri phenotype, containing 1 insertion of TL-DNA, and 2 shoot lines with a normal Bintje phenotype without TL-DNA. The 5th root clone showed no difference between sister shoot lines and the 6th root clone produced only 1 shoot line.

We conclude that during prolonged root culture and during shoot regeneration from root clones deletion of TL- and TR-DNA insertions can occur. The significance of the frequency of deletion of T-DNA of the Ri plasmid is discussed.

Introduction

Upon *Agrobacterium rhizogenes* infection of wounded parts of plants transformed roots develop, which contain part of the Ri plasmid (TL- and/or TR-DNA) [3, 22]. Transformed roots arise due to incorporation of TL- and/or TR-DNA in the genome of the plant cell and its expression induces root differentiation and growth. Transformed roots are often capable of regeneration into transformed plants [1, 20]. These plants show a deviant phenotype, the so-called Ri syndrome or hairy root syndrome, which is similar in many plant species.

Establishment of the full Ri syndrome is correlated with the expression of the *rol* A, B, C, and D loci located on the TL-DNA [18]. The products of these loci show synergistic activities, and

are involved both in rhizogenesis and in generating plant growth abnormalities [2, 17].

Previously we reported on plant regeneration from 7 Ri-transformed root clones of the potato cv. Bintje [6]. All 26 regenerated shoot lines were tetraploid. Phenotypic variation, observed among Ri plants appeared to be mainly root clone-dependent, particularly for height of plants, tuber size and tuber yield. In addition, segregation of phenotypic characteristics was found among the regenerants of 1 root clone, resulting in 2 plants with a normal Bintje phenotype and 1 plant with an Ri phenotype.

These results led to the following questions:

– Is the phenotypic variation due to the number of T-DNA copies present?

– Is loss of Ri phenotype due to loss of T-DNA or to inhibition of expression of T-DNA?

To this end, the presence, length and number of insertions of TR-and TL-DNA was determined in 16 regenerants from 6 root clones. Deletion of T-DNA was observed in sister shoot lines regenerated from 4 of the 5 root clones with more than 1 regenerant.

Materials and methods

Plants

Most root and shoot lines used have been described previously [6]. 16 Shoot lines regenerated from 6 transformed root clones of the tetraploid *S. tuberosum* L. cv. Bintje (Table 1) were obtained from infection with *A. rhizogenes* agropine strains AR15834 and LBA9402 (a derivative of AR1855).

From 1 root clone (*c*) 3 additional shoot lines B, C and D were regenerated after prolonged root culture for 2.5 years, on Murashige and Skoog (MS) medium [11]. Shoot lines originating from the same root clone are designated sister shoot lines.

Table 1. Characterization of Ri-transformed shoot lines and potato cv. Bintje control plants used

Root clone	Shoot lines[1]	Chromosome number[2]	Phenotype[3]		Tuber yield (g)
			shoot	tuber	
a	9; 10[4]	48	Ri	–	–
b	11; 13; 15; 16	48	Ri	Ri	5–10
c	19; 20;	48	1/2 Ri	Ri	50–60
	B, C, D	48			
d	22; 23	48	N	N	85–95
	24	48	N	Ri	27
f	25	48	1/2 Ri	Ri	18
g	31.2; 34	48	Ri	Ri	1–6
	Bintje	48	N	N	80–100

[1] Shoot lines were regenerated after 0.5 years except shoot lines B, C and D (after 2.5 years of culture).

[2] From chromosome counts in root tip cells [6].

[3] Expression of .most (Ri), some (1/2 Ri) and none (N) Ri characteristics.

[4] Both shoot lines did not grow in soil.

Opine tests

The presence of agropine and mannopine in both root and leaf tissue was established by paper electrophoresis and silver staining according to Petit *et al.* [14].

Southern hybridization of genomic DNA

Shoot or leaf material was taken from axenically grown shoots (14–21 days old) and from young growing plants (4–6 weeks after potting). Plant DNA was isolated using the method of Dellaporte *et al.* [4]. 10 μg DNA was digested with *Bam*HI, *Eco*RI, or *Hin*dIII (Boehringer, Mannheim, FRG) and electrophoresed on agarose gels. Southern blotting and hybridization were performed on GeneScreen Plus according to the manufacturer's manual (Du Pont Company, Boston, MA, USA). Probes were obtained by nick translation according to Maniatis *et al.* [10]. The probes used were pMP161, pMP101 and pMP66 covering TL-DNA of the Ri plasmid and pMP27 covering TR-DNA of the Ri plasmid (see Fig. 1A and 2A) for TL- and TR-DNA, respec-

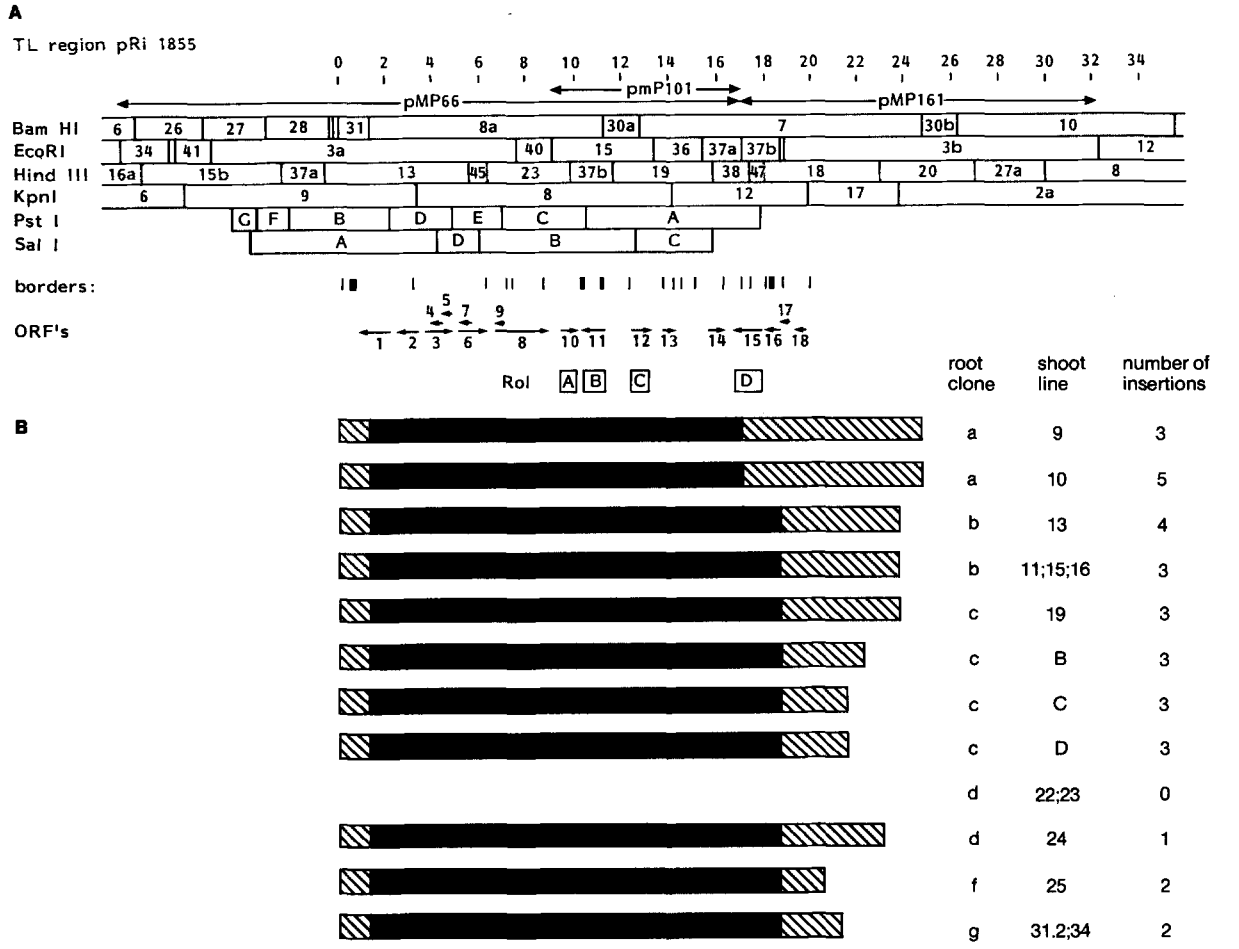

Fig. 1. A. Map of TL region on agropine type Ri plasmid according to Peerbolte [13]. The fragment numbers are according to Pomponi *et al.* [15], Jouanin [8], Huffman *et al.* [7] and De Paolis *et al.* [5]. Position of regions homologous to the open reading frames (ORFs) are according to Slightom *et al.* [16], and the position of *rol* loci according to White *et al.* [23]. The TL-DNA probes pMP161, 66 and 101 are indicated on top. B. Size and number of insertions of the TL-DNA present in transgenic potato plants. Solid bars indicate restriction fragments that are internal and striated bars indicate border fragments.

tively) [15] and were kindly supplied by Dr H.C. Hoge (State University, Leiden). Hybridizing DNA fragments having the same length as DNA fragments predicted by the physical map were presumed to be internal fragments whereas other fragments were considered to represent junctions between plant DNA and T-DNA, here defined as border fragments. The length of these latter fragments indicate the maximum size of the T-DNA insertion. The copy number was also estimated by comparison of the intensity of bands corresponding to internal fragments with reconstructions. All experiments were performed at least twice to make sure that the DNA had been fully digested.

Results

Number and length of insertions of TL-DNA

The number of TL-DNA insertions present in the plant genome, resulting from independent integration events and/or rearrangements was determined.

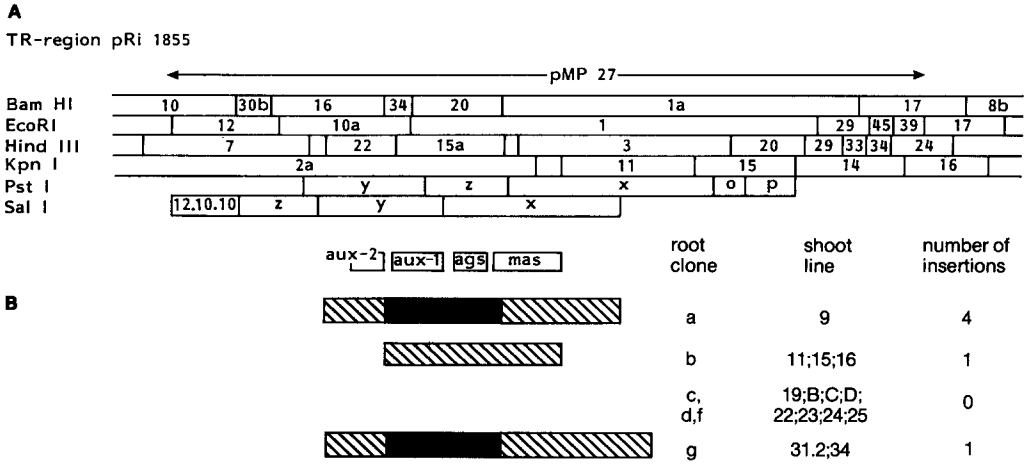

Fig. 2. A. Map of TR region on agropine type Ri plasmid. The origins of fragment numbers are given in the legend to Fig. 1A. The positions of regions homologous to known pTi genes are indicated: *aux*1 and *aux*2 loci according to Offringa *et al.* [12] and agropine (*ags*) and mannopine (*mas*) loci according to De Paolis *et al.* [5]. The TR-DNA probe pMP27 is indicated on top. B. Size and number of insertions of the TR-DNA present in transgenic potato plants. Solid bars indicate restriction fragments that are internal and striated bars indicate border fragments.

Examples of the hybridization pattern of DNA digests of the shoot lines from 3 root clones are given in Fig. 3. The pattern obtained with the 2 shoot lines of clone *g* (*g*34 and *g*31.2) is identical, containing the *Bam*HI fragments 8a and 30a together with 2 right border fragments (Fig. 3a). The pattern obtained with the 2 shoot lines from root clone *a* are different. Besides the internal

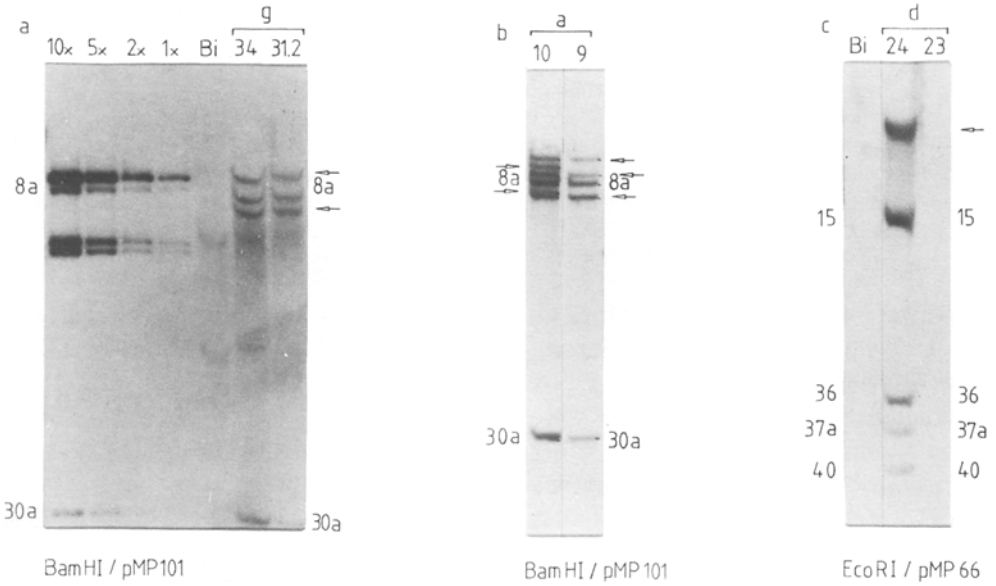

Fig. 3. TL-DNA hybridization pattern of DNA digests of transgenic potato plants, cv. Bintje and of Ri plasmid: shoot lines *g*31.2 and *g*34 from root clone, *g*, cv. Bintje and Ri-plasmid (a) and shoot lines *a*9 and *a*10 from root clone *a* (b) were hybridized with pMP101. Shoot line *d*23 and *d*24 of root clone *d* and cv. Bintje (*c*) were hybridized with pMP66. Border fragments are indicated with arrows.

fragments 8a and 30a line *a*9 contains 3 right border fragments while line *a*10 has besides these 3 2 additional ones (Fig. 3b). From root clone *d* shoot line *d*24 contains 4 *Eco*RI internal fragments (15, 36, 37a and 40) plus 1 left-border fragment, whereas shoot line *d*23 did not show hybridizing fragments (Fig. 3c). The number of insertions estimated was similar for each of the 3 restriction enzymes and probes used. Moreover, the copy number estimated by reconstruction experiments was in agreement with the number estimated by determination of border fragments. The results are summarized in Fig. 1b. In 3 out of 5 cases, the number of TL-DNA insertions found in sister shoot lines differed.

TR-DNA and opines

The presence and number of insertions of TR-DNA was analysed as described for TL-DNA. In shoot lines derived from root clones *c*, *d* and *f* no TR-DNA could be detected. *Bam*HI fragments (34 and 20) and *Hin*dIII (15a) were present in shoots from root clones *a* and *g*, while no internal fragments were found in the lines derived from root clone *b* (Fig. 4).

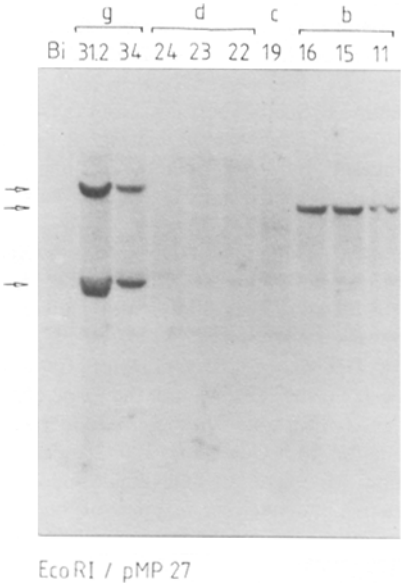

Fig. 4. TR-DNA hybridization pattern of transgenic potato DNA digests. Shoot lines *b*11, *b*15, *b*16 from root clone *b*, *c*19 from root clone *c*, *d*22, *d*23, *d*24 from root clone *d*, *g*31.2, *g*34 from root clone *g* and cv. Bintje *Eco*R1 digests were hybridized with pMP27.

Since TR-DNA is known to encode the enzymes for opine synthesis, all shoot lines were tested for the presence of opines. Agropine and mannopine were found in all shoot lines containing TR-DNA (data not shown). The absence of TR-DNA and opines in shoots derived from root clone *c* was remarkable since roots of this root clone produced opines at the time of shoot regeneration. Apparently, this root clone lost its TR-DNA between 2.5 and 3 years of subculturing.

Discussion

Presence of T-DNA

From the results presented above it is clear that sister shoot lines can contain different numbers of TL-DNA insertions. Previously it was found that sister shoot lines showed the same phenotype (ref. 6 and Table 1). Apparently, loss of some of the TL-DNA insertions did not visibly affect the phenotype of shoot lines with more than 1 insertion, indicating that not all insertions contributed equally to the expression of the Ri character. As expected, the Ri phenotype of shoot line *d*24 (with 1 copy) disappeared in *d*22 and *d*23 (no T-DNA).

The variation in number of insertions between sister shoot lines might be attributed to a possible chimaeric origin of root clones, to differential loss or to changes in T-DNA during root culture and plant regeneration. The presence of identical border fragments in sister shoot lines argues against a chimaeric origin. Loss of TL-DNA copies is more likely than amplification with rearrangements because

- deletion of a single copy of TR-DNA in root clone *c* was observed;
- deletion of a single copy of TL-DNA in 2 shoot lines of root clone *d* was observed;
- a molecular mechanism for deletion of DNA is more simply to envisage than for extra insertions.

An argument against variation of TL-DNA insertions by deletion is that from the 4 sister shoot lines of root clone *b* only one shoot line had 4 insertions while the other three all had the same 3 insertions. This might occur when at a certain locus one insertion is lost easily, or less likely lost during root culture resulting into the sampling distribution observed.

Deletion of T-DNA cannot be attributed to loss of chromosomes, known to occur frequently in dedifferentiated tissues in culture, since all shoot lines contained 48 chromosomes. However, it might be the result of mitotic cross over as shown by Loh *et al.* [9] in tomato.

Whether the frequent changes in TR- and TL-DNA are due to properties of the Ri T-DNA, to the plant species, or to the tissue culture methods used remains to be elucidated. Whatever the explanation may be, our results on the frequent loss of TL- and TR-DNA support the potential use of *A. rhizogenes* as a suitable tool for the introduction of desirable genes in recalcitrant crops, in addition to its high frequency of transformation, virulence for many crops, plant regeneration capacity, and maintenance of genetic stability of the plant genotype during root culture and plant regeneration [6, 19, 21].

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