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Structure of T-DNA in plants regenerated from roots transformed by *Agrobacterium rhizogenes* strain A4

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Summary. The structure of the T-DNA in Ri-transformed plants of *Brassica napus*, *Nicotiana plumbaginifolia* and *Nicotiana tabacum* was analysed. All the plants studied present a particular phenotype with wrinkled leaves. The T-DNA is composed of two parts: TL and TR. The size of the TL-DNA (19–20 kb) seems to be almost constant, except in *N. tabacum* where it is shorter. The TR-DNA can be absent, and its size varies from about 5–28 kb, with two predominant lengths. The smaller size does not include the region homologous to the *tms* genes of the pTi T-DNA. The copy number varies from one to four copies per plant genome. TL and TR-DNA are not always present in the same copy number, but in some cases are linked together.

Key words: *Agrobacterium rhizogenes* – Genetic transformation – Regenerated Ri transformed plants – Ri T-DNA – Size and copy number of Ri T-DNA

Introduction

The hairy-root syndrome is the result of a natural transformation event due to the bacterium *Agrobacterium rhizogenes*. The roots which appear at the inoculation point are genetically transformed: they contain part of the Ri plasmid (T-DNA) (Chilton et al. 1982; Willmitzer et al. 1982; Spano et al. 1982; White et al. 1982; Tepfer 1983, 1984). Transformed roots are able to regenerate into transformed plants (Ackermann 1977; Tepfer 1983; Costantino et al. 1984; David et al. 1984; Taylor et al. 1985; Guerche et al. 1987). Up to now, very few transformed roots and regenerated plants have been analysed for the precise structure of their T-DNA. In plants regenerated from roots transformed by agropine-type *A. rhizogenes* strains such as A4, two regions of the Ri plasmid (TL and TR) were reported to be present (Durand-Tardif et al. 1985; Jouanin 1984; White et al. 1985; Taylor et al. 1985). In Guerche et al. (1987), we describe a method to regenerate transformed rapeseed plants and in this paper we study the precise structure of the Ri T-DNA in these plants and in some additional transformed plants of *Nicotiana tabacum* and *N. plumbaginifolia*.

Materials and methods

Plants. Nicotiana plumbaginifolia was an anther-culture-derived haploid obtained by Goujaud and Bourgin (unpublished results); plants were cultured in sterile conditions. *Nicotiana tabacum* (var. Xanthi line XHFD8) was an anther-culture-derived haploid that had been diploidized in tissue culture (Bourgin 1978) and grown in the greenhouse. *Brassica napus* (oilseed rape, var. Brutor) seeds were germinated and grown in the greenhouse.

Inoculations. Plant inoculations were performed as described for tobacco by Tepfer (1984); roots appeared 3–5 weeks after inoculation.

Root cultures. Roots were made free of *A. rhizogenes* by several transfers on medium containing 300 μg/ml cefotaxime (a generous gift of Roussel-Uclaf) and cultures derived from a single root were established.

Regenerations. The transformed roots obtained after inoculation of *N. tabacum* and *N. plumbaginifolia* spontaneously regenerated plantlets. Cultures of rapeseed roots were induced to regenerate plantlets as described by Guerche et al. (1987).

DNA extractions. The plant DNA was extracted from 1 g fresh green leaves by the method of Dellaporta et al. (1983) slightly modified as a CsCl gradient centrifugation in a vertical rotor (Kontron TVT 65.5), in the presence of a small quantity of ethidium bromide (EtBr) to visualize the DNA, was performed after the first precipitation using the following conditions: refractive index, 1.3825; speed 60000 rpm, time, 4 h minimum. After the centrifugation, the single DNA band was recovered, the EtBr was removed and thereafter the DNA was diluted three times and precipitated by ethanol at $-20°$ C.

The plasmid DNAs used as probes were prepared by the Birnboim and Doly (1979) procedure followed by a CsCl-EtBr gradient (refractive index, 1.3925, speed 60000 rpm, overnight), after which the plasmid DNA was treated as described for plant DNA.

Digestions and electrophoresis. Generally 10 μg of plant DNA were digested for 5 h in medium salt buffer (Maniatis et al. 1982) with the appropriate restriction enzyme (*Bam*HI, *Eco*RI or *Hin*dIII) using 4 units per μg plant DNA in a final volume of 200 μl. The digestion was checked on aliquots (2 μl) on horizontal mini-gel, and was concentrated. Electrophoresis of plant DNA fragments was performed on vertical 0.8% agarose gels in TEA buffer (40 mM Tris-Acetate pH 8.0, 2 mM EDTA). After photography in the presence of a graduated ruler, the gels were denaturated as described in Maniatis et al. (1982), then the DNA fragments were transferred onto Amersham Hybond

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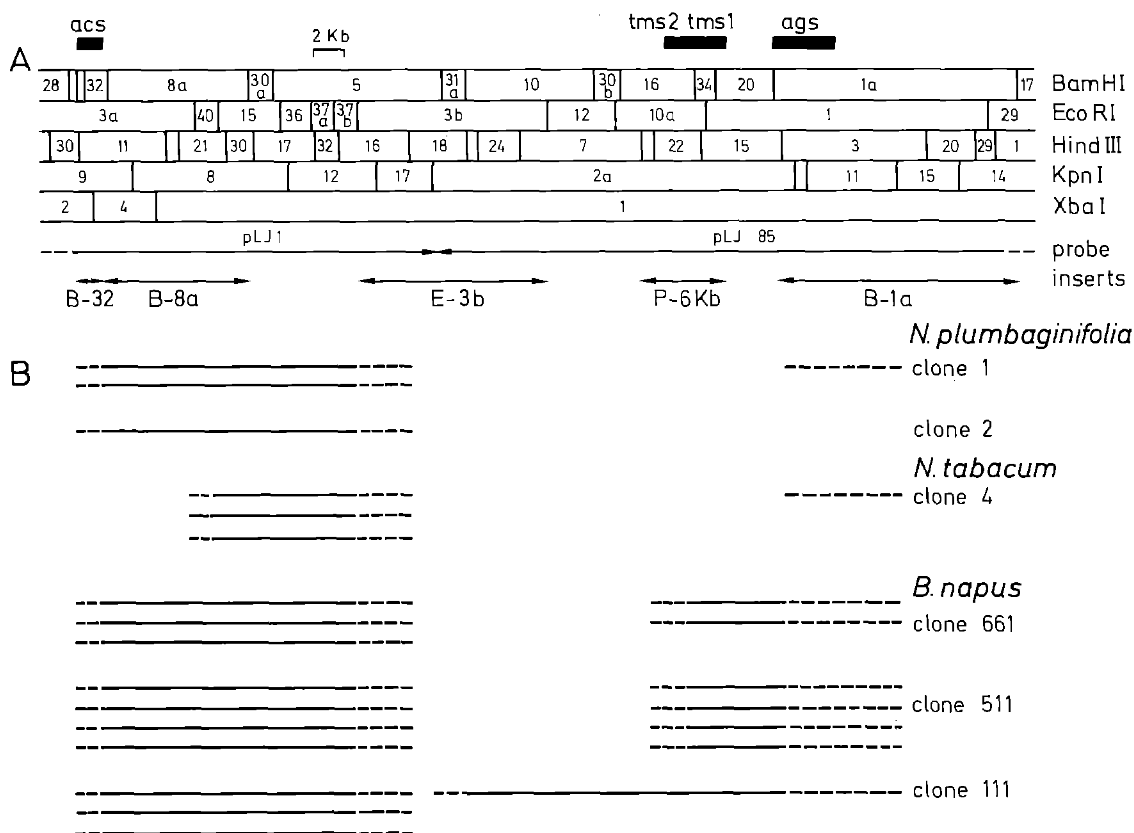

Fig. 1. A Map of the T region of pRiA4. The fragment numbers are from the complete map established by Jouanin (1984) for the enzymes *Bam*HI , *Eco*RI, *Kpn*I and *Xba*I, and by Huffman et al. (1984) for *Hin*dIII. The location of regions homologous to known pTi genes (Leach 1983; Jouanin 1984; Huffman et al. 1984; de Paolis et al. 1985) are indicated: acs, agrocinopine synthase; tms, genes involved in auxin synthesis; ags, genes involved in agropine synthesis. **B** A schematic representation of the size and copy number of the T-DNA transformed plants. *Solid bars* indicate restriction fragments that are internal and *open bars* indicate border fragments

nylon membrane as recommended by the manufacturer, using 20 × SSC as blotting buffer (1 × SSC is 150 mM NaCl, 15 mM Na citrate, pH 7). After washing (2 × SSC), the membrane was dried and the DNA was fixed by UV-irradiation. The nylon membrane (Amersham) can be re-used after elimination of the fixed radioactive probe by boiling the membrane for 15 min in 0.1% sodium dodecylsulfate (SDS).

Hybridizations. Plasmid DNA (1 µg) was labelled by nick-translation, using the Amersham kit and 100 µCi ^{32}P-dCTP. The hydridization conditions described in Maniatis et al. (1982) were used (6 × SSC, 68° C, 48 h). Filters were autoradiographed on Kodak XAR-5 films with intensifying screens at −70° C for 2–7 days.

Results

Choice of the probes and restriction enzymes

The restriction map of the T-regions of pRiHRI is shown in Fig. 1A. The probes used in the Southern blot experiments are indicated under the map and in Table 1. Two hybrid cosmids containing large parts (about 40 kb) of pRiHRI (identical to pRiA4 in the T-regions) were first used as probes: they are pLJ1 covering the TL region and pLJ85 covering the TR region (Jouanin 1984). *Bam*HI digests of plant DNA were first studied; *Eco*RI and *Hin*dIII digests of plant DNA were then used to identify precisely the T-DNA. Fragments of the T region entirely included in the plant genome (internal fragments) co-migrated with fragments of pRiHRI digests, while the other hybridizing fragments were considered as border fragments. To verify this interpretation and to determine the border fragments, smaller probes consisting of subcloned parts of the T regions were hybridized onto the same nylon filters; this allowed an easier interpretation of the results and led to a good estimate of the organization, the size and the copy number of the transferred DNA in the plants studied.

T-DNA in Nicotiana plumbaginifolia transformed clones

In *N. plumbaginifolia* clone 1 with probe pLJ1, the bands observed correspond to the pRi fragments *Bam*HI-8a, and 30a and to the fragments *Eco*RI-40, 15, 36, and 37a and b (Fig. 2a, e). The other bands are border fragments. With probe pLJ85, only one band is observed in each pattern (Fig. 2c, f), and it corresponds to TL border bands already observed (F on the *Bam*HI digest and F′ on the *Eco*RI digest). F is observed with probe pHSG262 (*Bam*HI-1a) and with probe pBR322 (*Eco*RI-3b) (Fig. 2c, d). We conclude that in this clone, two copies of TL-DNA are present, they lie from fragment B-8a to fragment E-37b. One copy of TR-DNA is observed, it includes only part of fragment B-1a. One TL copy and the TR copy are probably linked together as a border fragment of the same size is observed with both the TL and the TR regions as probes. Slightly hybridizing bands (X) are sometimes observed, they are attributed to "cellular T-DNA" (White et al. 1983), as they are also observed with DNA of normal *N. plumbaginifolia*

Table 1. Characteristics of the plasmids used as probes

Names	Size	Resistance	pRiHRI fragments	Source or reference
pLJ1	39.1 kb	Kan	B-32 to B-5	Jouanin (1984)
pLJ85	40.1 kb	Kan	B-30b to B-Ia	Jouanin (1984)
pBR322 (B-8a)	14.1 kb	Ap	B-8a	Leach (1983)
pBR322 (E-3b)	17.7 kb	Ap, Tet	E-3a	Vilaine (unpublished results)
pBR322 (P-6 kb)	10.3 kb	Tet	Pst 6 kb fragment	Vilaine (unpublished results)
pHSG262 (B-1a)	19.3 kb	Kan	B-1a	This work
pBR322 (B-32)	5.65 kb	Ap	B-32	This work

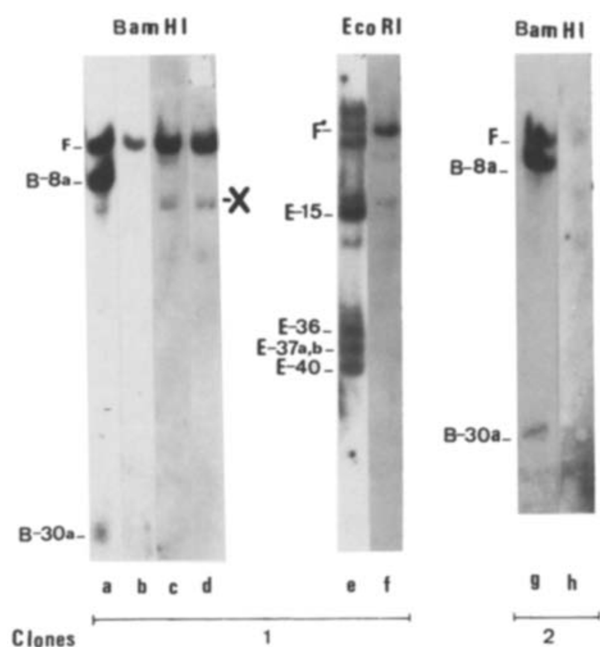

Fig. 2. Hybridization of total plant DNA digests of *N. plumbaginifolia* clones 1 and 2 with probes pLJ1 (*a*, *e*, *g*), pBR322 (E-3b) (*b*), pLJ85 (*c*, *f*, *h*) and pHSG262 (B-1a) (*d*). The internal fragments are indicated by their fragment numbers on pRi and some border bands are denoted F and F′

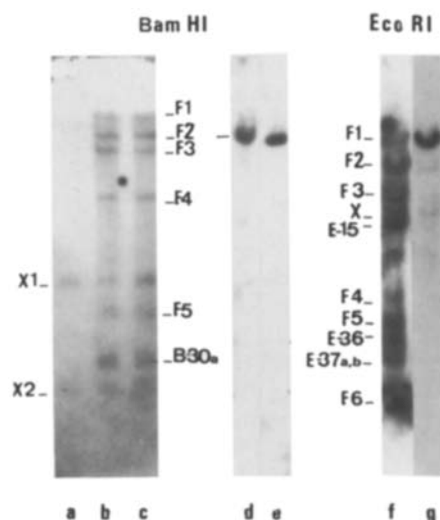

Fig. 3. Hybridization of tobacco DNA digests (a, normal plant; b–g, transformed clone 4) with probes pLJ1 (*a*, *b*, *c*, *f*), pLJ85 (*d*, *g*) and pHSG262 (B-1a) (*e*). The internal fragments are indicated by their pRi fragment number and the border bands denoted F1–F6

(not shown). Hybridizations of digests of total DNA from *N. plumbaginifolia* clone 2 against pLJ1 reveal the same internal bands as clone 1. With probe pLJ85, no hybridization is observed (Fig. 2h). *N. plumbaginifolia* clone 2, therefore possesses a single copy of TL-DNA and no TR-DNA.

The *Bam*HI fragment of transformed plant DNA containing the left TL border is only observed after a very long exposure using pBR322 (*Bam*HI-32) as probe (not shown), this was observed already in the analysis of transformed *Convolvulus arvensis* (Leach 1983), and appears to be the case for all the plant DNA *Bam*HI patterns studied so far, except transformed tobacco.

T-DNA in clone 4 of tobacco

Durand-Tardif et al. (1985) studied the expression of the Ri T-DNA in transformed tobacco and the presence of the T-DNA in two clones (clones 4 and 9). Clone 4 is analysed here more precisely as it was used in the laboratory for further experiments (Tourneur et al. 1985). The TL-DNA of clone 4 is composed of the fragments B-30a, E-15, E-36, E-37a and b, (Fig. 3b, f). With probes pLJ85 and pHSG262 (B-1a) only F2 (15 kb) was observed (Fig. 3d, e). Bands which hybridized slightly with pLJ1 can often be observed, they are, however, also present in patterns of normal tobacco DNA (X1 and X2, Fig. 3a). These bands are attributed to cT-DNA (cellular T-DNA), already described as present in some normal plants (White et al. 1983). Clone 4 of transformed tobacco contains three copies of TL-DNA lying from E-15 to E-37b, and one copy of TR-DNA including only a part of fragment B-1a. As in *N. plumbaginifolia* clone 1, one TL and the TR copies are linked together. No change of the T-DNA was observed when plants of the progeny showing the typical hairy-root phenotype were analysed (Fig. 3c).

T-DNA in transformed oilseed rape clones

In oilseed rape clone 661 (and 741), the TL-DNA contains the same internal fragments as those observed in the *N. plumbaginifolia* clones 1 and 2. The other bands, in *Bam*HI digests, (F1, F2, F3) contain borders, and are identified using probe pBR322 (*Eco*RI-3b) (Fig. 4b). With probe pLJ85, the fragments B-20, B-34 and H-15 are shown to be internal (Fig. 4c, f, h) and in *Bam*HI digests, three border fragments (F1, F3, F4) are observed. Two of them (F1 and F3) are also observed with probe pHSG262 (B-1a) (Fig. 4d). Thus, clone 661 contains three copies of the TL-DNA and two copies of the TR linked with two right border fragments of the TL-DNA copies. The border fragments F1 and F3 include part of E-3b (in B-5) and part of B-1a; this observation leads to the conclusion that the TR copies in the plant DNA must be in a reverse position, compared

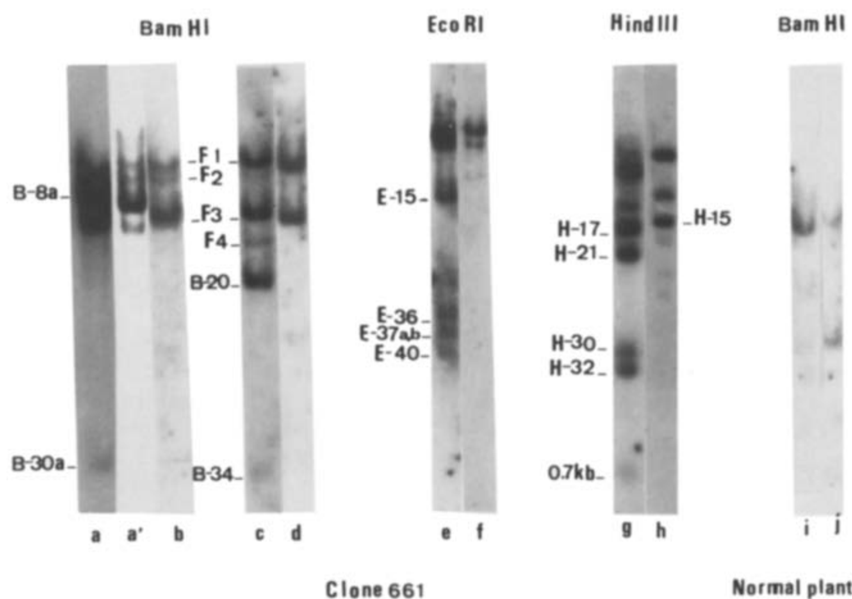

Fig. 4. Hybridization of total DNA digests of clone 661 (*a-h*) and of normal oilseed rape (*i, j*) with probes pLJ1 (*a*, *a'*, a with a shorter exposure time, *e*, *g*), pBR322 (E-3b) (*b*, *i*), pLJ85 (*c*, *f*, *h*) and pHSG262 (B-1a) (*d*, *j*). The internal fragments are indicated by their pRi fragment number and the *Bam*HI borders denoted F1–F4

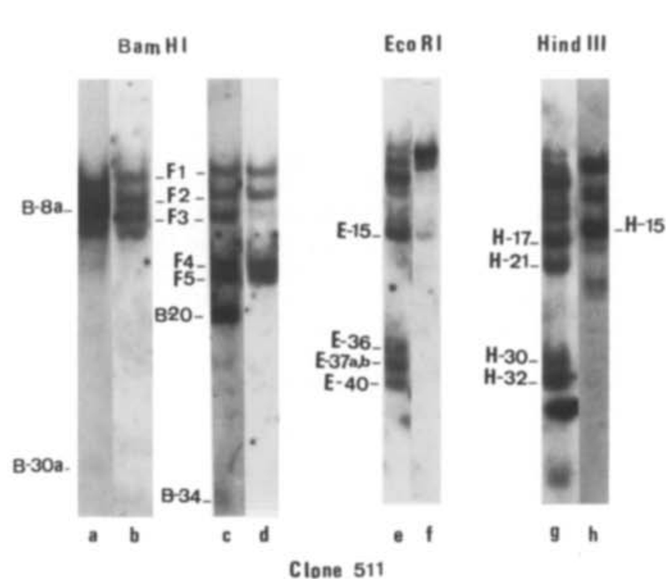

Fig. 5. Hybridization of oilseed rape clone 511 total DNA digests with probes pLJ1 (*a*, *e*, *g*), pBR322 (E-3b) (*b*), pLJ85 (*c*, *f*, *h*) and pHSG262 (B-1a) (*d*). The internal fragments are denoted by their pRiHRI fragment number and *Bam*HI border fragments denoted F1–F5

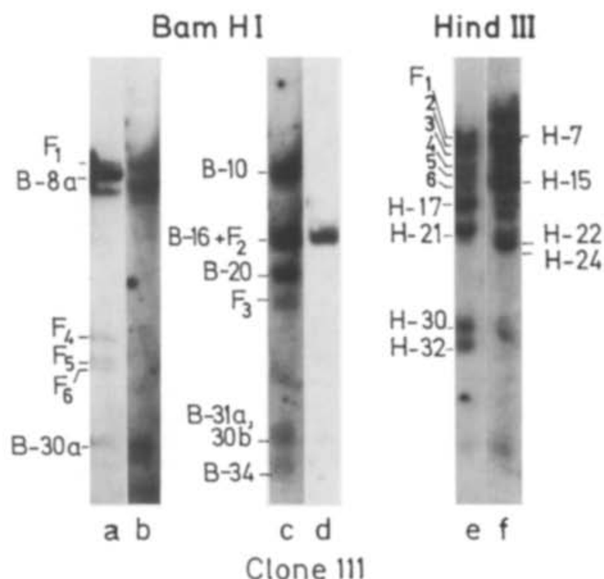

Fig. 6. Hybridization of oilseed rape clone 111 total DNA digests with probes pLJ1 (*a*, *e*,), pBR322 (E-3b) (*b*), pLJ85 (*c*, *f*) and pHSG262 (B-1a). The internal fragments are denoted according to their pRi fragment number and *Bam*HI and *Hin*dIII border fragments denoted F1–F6

to the orientation of the TR region relative to the TL region in the plasmid.

In clone 511, the sizes of the TL and TR-DNA are the same as in clone 661. On *Bam*HI digests at least five border fragments (F1, F2, F3, F4, F5) are observed. Two of these fragments (F1, F2), are also observed with probe pHSG262 (B-1a) (Fig. 5c, d). In this clone, there are probably four copies of TL and TR-DNA, two of them are linked in the same manner as those found in clone 661.

In clone 111 (and 233), the same internal TL-DNA fragments found in the two former clones are observed with probe pLJ1 (Fig. 6a, e). Probe pLJ85 reveals bands corresponding to fragments B-31a, B-10, B-30a, B-16, B34, B20 and part of fragment B-1a (Fig. 6c, d). Clone 111 contains three copies of TL-DNA of the same size as the other rapeseed clones and at least one copy of TR-DNA which is longer than in the other clones studied.

One band of 8 kb is observed in *Bam*HI pattern of normal rapeseed DNA with probes pLJ1, pHSG262 (B-1a) and pBR322 (E-3b) and with pHSG262 (B-1a) another band of 3.5 kb is observed (Fig. 4i and j). These bands are only observed after a long exposure and must be attributed to cT-DNA as in tobacco (White et al. 1983).

Discussion

A schematic representation of the size and of the copy number of the T-DNA in the studied transformed plants is shown in Fig. 1B. Except tobacco where the TL-DNA is shorter on the left side, all the studied transformed plants

possess a TL-DNA of the same size. A TL-DNA of this size is present in *Convolvulus arvensis* clone 7. A genomic library of the DNA of this clone has been constructed (Slightom et al. 1985) and recombinant phages, including the plant DNA/plasmid DNA junctions have been selected and sequenced. The exact size of the TL-DNA determined from the nucleotide sequence is 19,416 bp (Slightom et al. 1986). In this *C. arvensis* clone, the TR-DNA extends from inside fragment B-16 to inside fragment B-1 a (approximately 12 kb), and TL and TR regions are linked (unpublished results). This is observed in some other clones of *N. plumbaginifolia*, oilseed rape and tobacco, but is not a general feature, as the copy number of TL- and TR-DNA can be different. The size of the TR-DNA is variable, it can either be absent or consist of only part of fragment B-1a, or include also fragments B-20, B-34 and part of fragment B-16. In one clone of oilseed rape, the TR-DNA is longer; this result was also observed in a *C. arvensis* clone (unpublished results). The size of the TL-DNA in transformed *N. plumbaginifolia* and oilseed rape are consistant with those already obtained in transformed *C. arvensis*, carrot, *Brassica oleacera* and *Atropa belladonna* (unpublished results and D. Tepfer, personal communication).

The 25 bp direct repeat found on the plasmid TL region, at positions 520 and 19966, near the extremities of the TL-DNA (Slightom et al. 1985, 1986) probably explains the size of the TL-DNA in these plants. Tobacco seems to be an exception as it is the only plant where the observed TL-DNA is always shorter on the left, as shown here for clone 4, by Durand-Tardif et al. (1985) for clone 9 and by Taylor et al. (1985). Inside the TL region of pRiA4 the presence of 25 bp sequences homologous to the pTi 25 bp consensus sequence (Slightom et al. 1986) can perhaps explain the size of the TL-DNA in transformed tobacco.

The presence of the 25 bp direct repeats at the extremities of the T region of pTi are required for the transformation procedure (Shaw et al. 1984). Deletion of the right border sequence abolishes tumour formation (Wang et al. 1984; Peralta and Ream 1985) and deletion of only the left border lowers it (Peralta and Ream 1985). In plant transformation with pRiA4, the right border of the TL-DNA seems to be more constant than the left one, so their relative importance in pRi is perhaps similar to those of pTi. The presence of such sequences in the TL region of Ri plasmids shows their generality in *Agrobacterium*, and the presence of several of these sequences in the pRi TR region may explain the different sizes of the TR-DNAs.

The TL region of pRiA4 does not share homology by hybridization (Jouanin 1984; Huffman et al. 1984) and by comparison of nucleotide sequences (Slightom et al. 1986) with the TL region of octopine pTi (Barker et al. 1983; Gielen et al. 1984). In plants transformed by the Ri plasmid of *A. rhizogenes* strain 8196 (which presents no homology with pTi *tms* genes, Lahners et al. 1984) only one region is observed to be transferred (Byrne et al. 1983) and this region is very homologous to the TL region of pRiA4 (Spano et al. 1982; Leach 1983).

Analysis of the results presented here shows that the TR-DNA size is more variable (0 kb to about 28 kb). It was already shown (Willmitzer et al. 1982; Jouanin 1984; Huffman et al. 1984) that the TR region of pRiA4 contains homologies with pTi T-DNA genes such as the *tms* 1 and *tms* 2 genes (Garfinkel et al. 1981; Ooms et al. 1981; Lemmans et al. 1982; Joos et al. 1983) of the conserved region and the agropine synthesis genes of the octopine TR-DNA (Velten et al. 1983; Salomon et al. 1983). The minimum part of the TR-DNA, when present, is composed of the left part of fragment *Bam*HI-1 a; within this region are located the genes involved in agropine synthesis (De Paolis et al. 1985; D. Bouchez, personal communication). Only transformed oilseed rape clones synthesize mannopine, but mannopine and agropine are rarely observed in regenerated tobacco and *C. arvensis* plants (Tepfer 1984), thus this region can be present and not expressed in transformed plants.

The region that shares homology with the *tms* genes of Ti T-DNA is not always present in transformed plants: it is present in oilseed rape clones and in *C. arvensis* clones 7 and 10 (unpublished results) and absent in *N. plumbaginifolia* and tobacco. Similar observations were reported by Durand-Tardif et al. (1985) and Taylor et al. (1985). In Ti T-DNA, this region is involved in auxin synthesis (Inze et al. 1984; Schroder et al. 1984). If, like those of Ti T-DNA, these genes are functional in transformed roots, they must upset the hormonal balance, and can be incompatible with regeneration in species such as *N. tabacum* and *N. plumbaginifolia*. TR-DNA can be absent in the transformed plants, so TL-DNA alone seems to be able to induce the formation of roots, and then to regenerate plants. Strains carrying plasmids deleted of TL or TR regions are both able to induce transformed roots but with different intensities of response according to the species (Vilaine and Casse-Delbart 1987).

Durand-Tardif et al. (1985) studied the expression of the T-DNA in clone 9 of tobacco. Only transcripts corresponding to the TL-DNA were observed. The hairy-root phenotype must be due to genes located on the right side of the TL-DNA, as in tobacco this region is devoid of its left part, and because *N. plumbaginifolia* clone 2 and a clone of *Brassica oleacera* (unpublished results) without TR-DNA share the typical hairy-root phenotype.

The copy number of the Ri T-DNA is low, it varies according to the plants studied from one to four copies of TL-DNA, and from zero to four copies of TR-DNA. The copy number of TL- and TR-DNA can be different and, in some instances, one copy of TL-DNA and one copy of TR-DNA are linked with an inversion of the TR with respect to its position in the plasmid. In clone 4 of tobacco no segregation of the copies of the T-DNA is observed in the progeny. The oilseed rape plants are fertile so the organization of the T-DNA of their progeny will be analysed. Since several copies are present, if they resulted from independent transformation events, segregation might be observed (Guerche et al. 1987 and in preparation).

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