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# Reversion of Aberrant Plants Transformed with *Agrobacterium rhizogenes* Is Associated with the Transcriptional Inactivation of the $T_L$-DNA Genes[1]

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## ABSTRACT

**Transgenic plants harboring the left transfer DNA ($T_L$-DNA) of the root inducing plasmid of *Agrobacterium rhizogenes* show many developmental abnormalities. We observed frequent appearance of normal looking lateral (revertant) shoots from such aberrant plants. Unlike aberrant shoots of the plant, revertant shoots exhibited a very high growth rate and set viable seeds. Sexual and vegetative reproduction studies showed inheritance of the revertant phenotype. Southern hybridization experiments demonstrated that the T-DNA pattern was identical in aberrant and revertant shoots, indicating that the revertant phenotype was not due to deletion or rearrangement of the T-DNA genes. Specific T-DNA transcripts were not expressed in revertant shoots. Thus, the revertant phenotype appears to result from the transcriptional inactivation of T-DNA genes. We propose that similar events in the past may have mediated horizontal acquisition of $T_L$-DNA genes by ancestors of the genus *Nicotiana*, which are still found as silent endogenous T-DNA in present day untransformed *Nicotiana* species.**

*Agrobacterium rhizogenes* incites tumor formation on many plants (7). These tumors are characterized by initial callus formation and subsequent extensive root proliferation. The virulence of *A. rhizogenes* is dependent on the large Ri[4] plasmid, a portion of which is transferred to and stably integrated into the plant genome (5, 28, 31; for recent reviews see Refs. 20, 29). The Ri plasmid present in the agropine type strain A4 contains two transferred DNA (T-DNA) regions which are separated by about 15 kb of nontransferred DNA (14, 30). The T-DNA transfer is probably mediated by border sequences similar to the Ti plasmid T-DNA borders (21). The T-DNA genes are transcribed as polyadenylated mRNA in the plant nucleus (8, 23, 31).

The right T-DNA region ($T_R$-DNA) of the Ri plasmid contains two genes involved in auxin biosynthesis referred to as *tms1* and *tms2* (also referred to as *Aux1* and *Aux2*, respectively; Fig. 1). These genes show extensive DNA sequence homology with the similar genes found on the Ti plasmids and mutations in these genes are interspecifically complemented (14, 17, 30). The *tms1* gene product, tryptophan monooxygenase, converts tryptophan to indoleacetamide which is then hydrolyzed by the gene product of *tms2*, indoleacetamide hydrolase, to IAA (19). The $T_R$-DNA also contains the genes for the synthesis of agropine (14). The left T-DNA ($T_L$) is about 20 kb in size and does not show any significant DNA homology with other Ti plasmids. The $T_L$-DNA has been sequenced and a total of 18 open reading frames have been identified (21).

Both T-DNA regions participate in root induction either individually or in concert depending on the plant species or tissues (26, 30). The *tms1* and *tms2* loci are essential for root induction on *Nicotiana tabacum* stems, basal side of carrot disks and *Kalanchoë diagremontiana* leaves (3, 26, 30). Similarly, four $T_L$-DNA loci (*rol*A, *rol*B, *rol*C, and *rol*D) have been identified by transposon mutagenesis that affect tumor phenotype on *K. diagremontiana* leaves (30; Fig. 1). In contrast to the *tms* loci, the function of the *rol* loci at the biochemical level are unknown.

Perhaps the most interesting observation made with the Ri T-DNA is the presence of $T_L$-DNA homologous sequences in untransformed plant species, notably in the genus *Nicotiana* (27, 28). A genomic clone of the homologous endogenous T-DNA was isolated and sequenced from *Nicotiana glauca*. The DNA sequence of the clone showed that the open reading frames and the intergenic sequences of the endogenous T-DNA are highly related (84% at DNA level and 75% at amino acid level) to the bacterial sequences (9). The sequence data also showed that the endogenous T-DNA exists as an inverted asymmetric repeat, and comparison of the sequence divergence between the two arms suggested that the endogenous T-DNA has been present in *N. glauca* for several million years—probably antedating speciation of the genus *Nicotiana*.

Plants containing T-DNA can be readily regenerated from Ri-transformed tissues (1, 6, 18, 22, 24). These transgenic plants exhibit several developmental abnormalities which are consistently observed in a variety of plant species (1, 18, 22, 24; VP Sinkar, unpublished data). These abnormalities include severely wrinkled leaves, loss of apical dominance, stunted growth, decreased root geotropism, and floral hyperstyly. The transformed phenotype has been associated with the $T_L$-DNA (22), and particularly with the *rol*A locus (VP Sinkar, unpublished data). We observed frequent reversion of such aberrant plants regenerated from transformed tissues. In *N. glauca*, normal looking lateral branches were formed from the basal axillary buds of the aberrant plants. Similar observations were also made with aberrant

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[4] Abbreviations: Ri, root-inducing; T-DNA, $T_L$, $T_R$, transfer DNA, left and right region.

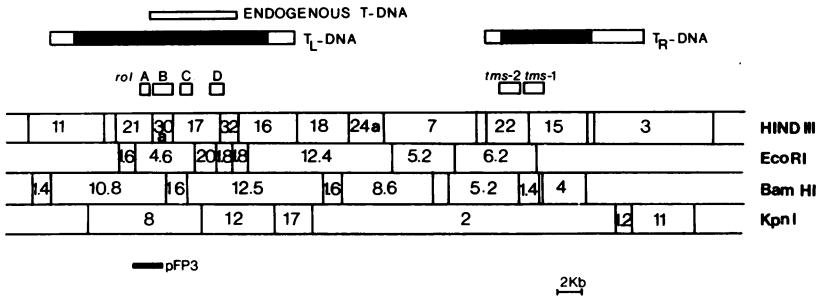


FIG. 1. Restriction map of the T-DNA of the pRiA4b. Positions of the *rol* loci as identified by transposon mutations are shown (30). Also shown are the positions of the *tms1* and *tms2* loci (14) and the extent of endogenous T-DNA present in *N. glauca* (9). Restriction fragment used to clone *rol*A locus (pFP3) is also shown. For more details please refer to the Table I.

*Nicotiana* tabacum plants transformed with the Ri plasmid or with *rol*A locus. We present evidence in this communication that the phenomenon of reversion is associated with the transcriptional inactivation of the $T_L$-DNA genes and not with detectable rearrangement of the $T_L$-DNA.

## Material and Methods

**Bacterial Strains and Plasmid.** *Agrobacterium rhizogenes* and *Escherichia coli* strains and plasmids used in the study are listed in Table I. *Agrobacterium* strains were maintained on AB minimal agar (30) supplied with the appropriate antibiotic when necessary. Antibiotic concentrations used were 100 $\mu$g/ml kanamycin (Sigma), 100 $\mu$g/ml carbenicillin (Geopen, Pfizer Ltd), and 100 $\mu$g/ml gentamycin (Sigma). *E. coli* strains were maintained on L-agar supplemented when appropriate with 100 $\mu$g/ml kanamycin, 100 $\mu$g/ml carbenicillin, 10 $\mu$g/ml tetracyclin (Sigma) or 50 $\mu$g/ml nalidixic acid (Sigma).

**Plant Inoculations, Tissue Propagation, and Regeneration.** Stems of axenically grown *N. glauca* var *Smith* plants were infected with *A. rhizogenes* A4. Tumors formed on the stem were excised and grown on Murashige and Skoog medium in the absence of added hormones ($MS^-$) (16). Unlike the tumors formed by *A. tumefaciens* these tumors showed tendency to produce roots. Such roots were excised from the tumors and were cultured on $MS^-$ medium where they frequently gave rise to shoots and eventually differentiated into well developed plants.

Discs obtained from leaves, between the fourth and sixth position from the apex, were used for the transformation of *N. tabacum* var *xanthi*. The discs were infected with *A. rhizogenes* R1500 (Table I) by the method described by Horsch *et al.* (13). Roots formed on the discs were excised and grown on $MS^-$ medium containing kanamycin (200 $\mu$g/ml). These roots frequently regenerated into plants.

To obtain transgenic plants containing *rol*A locus, *N. tabacum* leaf discs were infected with *A. tumefaciens* LBA 4404 (a helper strain which provides the *vir* functions *in trans*; 12) containing *rol*A locus (pFP3, VP Sinkar, unpublished data). The leaf discs were grown on MS 104 growth medium (13) containing kanamycin (100 $\mu$g/ml). Kanamycin resistant shoots formed from these discs were excised and grown on $MS^-$ medium supplemented with kanamycin.

Well differentiated plantlets were transferred to the mixture of vermiculite and peat moss (1:2) and grown in the plant growth room with 16 h light and 8 h dark cycle.

**Plant Nucleic Acid Isolation and the T-DNA Analysis.** Plant nucleic acids were isolated by modification of the extraction proccedure previously described (22). Plant DNA was digested with 3 to 4 units of the appropriate restriction enzyme for 4 h under the conditions specified by the manufacturer. The digested DNA was fractionated on agarose gel by electrophoresis. Gels were washed with 0.25 M HCl for 15 min at room temperature followed by 45 min treatment each with 0.5 M NaOH-0.8 M NaCl and 0.5 M Tris (pH 7.9)-15 M NaCl. The denatured DNA was transferred to nitrocellulose by capillary transfer as previously described (30). Nitrocellulose papers containing bound DNA were baked at 80°C for 2 h and prehybridized at 42°C overnight in 50% formamide-6X SSC (1XSSC = 0.15 M NaCl, 0.015 M Na citrate) -50 mM $NaH_2PO_4$ (pH 7.0)-12.5 mM EDTA-5X Denhardt solution (1x Denhardt = 0.02% bovine serum albumen, 0.02% Ficoll, 0.02% polyvinyl-pyrrolidine)-0.5% Na-dodecyl sulfate-100 g/ml denatured salmon sperm DNA-10% dextran sulfate. Nick translated probes labeled with $^{32}P$ were denatured and added to the same mix to obtain $1 \times 10^6$ cpm/ml. Hybridization was carried out for 36 h at 42°C, the filters were then washed with 2 X SSC-2.5 mM EDTA-0.1% SDS at room temperature for 15 min followed by two washes with 0.1 X SSC at 65°C for 30 min each. The washed blots were exposed to KODAK film XAR-2 with a Cronex intensifying screen.

RNA isolated from these plants was fractionated on an oligo-dT cellulose column to isolate poly($A^+$) mRNA, was size-frac-

Table I. *Bacterial Strains and Plasmids used in This Work and their Relevant Characteristics*

| Strains and Plasmids | Relevant Phenotype and Genotype | Source |
|---|---|---|
| *A. rhizogenes* | | |
| R1000 (pRiA4b) | $onc^+$ | Derivative of A4T (30) |
| R1500 | $onc^+$, $Carb^R$, plant $Kan^R$ in H 21 | Derivative of pRiA4b (VP Sinkar, unpublished data) |
| *A. tumefaciens* | | |
| LBA 4404 | $onc^-$, $Spec^R$ | Derivative of pTiAch5 (12) |
| *E. coli* | | |
| HB101 | *thr*, *leu*, *thi*, *pro* | |
| *Plasmids* | | |
| pFW 302 | $Tc^R$, $RK2^{rep}$, IncP1 | H-11 to H-18 of pRiA4b in pVK 102 (30) |
| pFP 3 | $Tc^R$ | *rol*A locus into pGA 472, a Ti plasmid derived vector for plant transformation (VP Sinkar, unpublished data) |

tionated on formaldehyde agarose gel and was blotted on onto nitrocellulose paper (22). Hybridization and blot washing conditions used were the same as described above.

## RESULTS

**Transgenic Plants and Reversion.** Transgenic plants containing the Ri $T_L$-DNA or *rol*A locus were aberrant in appearance. However, after about 4 to 10 months of growth in the plant growth room, normal looking lateral (revertant) shoots started appearing from basal axillary buds (Fig. 2, A–D). These shoots grew much faster than the abnormal shoots. The revertant shoots of *N. glauca* grew beyond 3 m in height within 6 months after their appearance while the aberrant shoots remained stunted at about 70 cm even after 12 to 16 months. In fact, in most of the plants the growth of the aberrant shoot halted after the appearance of the revertant shoots. In *N. glauca*, where the reversion was more clear-cut, immediately after the appearance of the revertant shoot the thickness of the main shoot (aberrant) diminished and leaf abscission was notable. Leaf replacement was either absent or very low while leaves on the revertant shoots were healthy and normal leaf regeneration was observed after abscission. In most plants the aberrant shoots eventually died or bore small, normal looking leaves indicating that the reversion process was complete. Leaf abscission and reduction in the size of the aberrant shoot was less evident in *N. tabacum*, although similar to the behavior observed in *N. glauca*, the revertant shoots grew very rapidly. Since the reversion appeared to be more distinct in *N. glauca* we decided to use these plants for further characterization of this phenomenon.

**Inheritance of Revertant Phenotype.** Vegetative propagation was carried out using leaves of aberrant and reverted *N. glauca*. The aberrant leaves formed roots on $MS^-$ medium, and these roots occasionally formed shoots. Mostly these shoots grew as teratomas while those well differentiated inherited the parental aberrant characters (Fig. 3A). The revertant leaves required both an auxin (naphthalene aceitic acid) and a cytokinin (6-benzylaminopurine) for growth and formed normal looking plantlets on the MS 104 growth medium (Fig. 3B). Sexual reproduction was successful only with the revertant shoots of the plants. The flowers on these shoots were self fertile and set viable seeds. Analysis of the $F_1$ progeny showed more than 85% normal plants (Fig. 3C), whereas the remaining progeny grew slowly, died, or showed aberrant phenotype. Flowers of the aberrant shoots were very small, about 45 to 50% in length and width (2.5–3 cm in length and 0.2–0.3 cm in width) compared to normal, and showed hyperstyly. The stamens of these flowers had a very low pollen count and repeated attempts at self-fertilization were unsuccessful.

**T-DNA Analysis.** All aberrant *N. glauca* plants obtained from one transformed tissue culture gave rise to revertant shoots within 8 months of growth in the plant room. We analyzed the revertant and aberrant shoots of three plants regenerated from this culture for $T_L$-DNA content by southern hybridization. DNA isolated from these shoots was digested with various cytosine methylation insensitive restriction enzymes and was probed with a cosmid clone containing the entire $T_L$-DNA. The same $T_L$-DNA pattern was detected in both revertant and aberrant shoots of all three plants, indicating that the revertant phenotype was not due to a deletion or rearrangement of the $T_L$-DNA sequences (data are shown for *Hin*dIII digestion, Fig. 4A). All internal T-DNA fragments, with the exeption of fragment 17 which has undergone an internal deletion, were seen in both types of shoots of the three plants. Since both normal and aberrant shoots have this deletion most probably it occurred at the time of transformation or before transformation in the bacterium and therefore it has no relevance to reversion. Further, the fragment 17 has been shown not to be involved in controlling the aberrant phenotype (VP Sinkar, unpublished data; also see data presented with transgenic *N. tabacum* plants in this paper). We also analyzed two transgenic *N. tabacum* plants, one transformed with the Ri plasmid (R1500, Table I) modified to contain a plant expressible kanamycin resistance gene in *Hin*dIII fragment 21 and the other with *rol*A locus alone. In both these cases, too, no deletion or rearrangement of the T-DNA was seen in the revertant shoots. As shown in Figure 4B both normal and revertant shoots of *N. tabacum* transformed with the modified Ri plasmid showed all internal T-DNA fragments, *i.e.* *Hin*dIII 17, 30a, and 32 with the exception of fragment 21. Fragment 21 is not present at its usual position because insertion of the plant expressible kanamycin gene cartridge has increased its mol wt and thus it migrates slowly (the intense band at the top). Similarly, an expected *Hin*dIII fragment of 3.2 kb indicative of the presence of *rol*A locus, which comprises part of the *rol*A locus and vector sequences, is present in both aberrant and revertant shoots of *rol*A containing *N. tabacum* (Fig. 4C). DNA obtained from the Ri and *rol*A transformed *N. tabacum* plants was also examined for the T-DNA pattern using other restriction enzyme digests. With all enzymes (methylation insensitive) used, the same T-DNA pattern was observed in the aberrant and revertant shoots of each plant (results shown only for *Hin*dIII).

**Transcript Analysis.** Since no loss or rearrangement of the T-DNA was seen in revertant shoots of any plants tested, we examined these plants for the T-DNA specific transcripts. RNA isolated from leaves of both revertant and aberrant shoots was size-fractionated on formaldehyde-agarose gel, blotted onto nitrocellulose, and probed with $T_L$-DNA specific probes. As shown in Figure 5 all the aberrant shoots of *N. glauca* expressed at least two RNA transcripts (0.8 and 2.8 kb) homologous to the $T_L$-DNA, whereas these transcripts were notably absent in revertant shoots. Some plant specific variations were observed in these studies; for instance, aberrant shoots of *N. glauca* regenerates 1 and 2 showed four transcripts (0.8, 1.2, 1.6, and 2.8 kb; Fig. 5A) homologous to the $T_L$-DNA while the aberrant shoot of the plant 3 expressed only two transcripts (0.8 and 2.8 kb; Fig. 5B) homologous to the $T_L$-DNA. The revertant shoots of the plants 1 and 2 still expressed 1.2 and 1.6 kb transcripts homologous to the $T_L$-DNA whereas no homologous transcripts were detected in the revertant shoot of plant 3. These observations indicate that the 0.8 and 2.8 kb transcripts, individually or together, control the aberrant phenotype. Further analysis showed that these transcripts hybridize only to *Hin*dIII fragments 21 and 30a of the $T_L$-DNA (data shown for plant 3, Fig. 5B; see Fig. 1 for the restriction map of the $T_L$-DNA). The sequence and genetic data indicate that the transcript emerging from the *rol*A locus would hybridize with both these fragments (33, 49), therefore it seems that the transcripts (at least the 0.8 kb) represent the *rol*A locus. Its association with the aberrant phenotype is consistent with our observations that the *rol*A locus controls the aberrant phenotype (VP Sinkar, unpublished data). We also performed $T_L$-DNA transcript analysis with one *N. tabacum* plant transformed with the Ri plasmid (R1500). Similar to those in *N. glauca* plants the revertant shoots of this plant showed absence of 0.7 to 0.8 kb T-DNA specific transcript, the only difference being that the 2.8 kb transcript was not detected in the aberrant shoots of this plant (data not shown).

## DISCUSSION

Transgenic plants containing $T_L$-DNA of *A. rhizogenes* exhibit aberrant features. These plants invariably undergo reversion, and normal looking new shoots appear from the basal axillary buds. In all plants examined we found that the revertant shoots still retained the $T_L$-DNA and no rearrangement or deletions were noticed. However, $T_L$-DNA specific transcripts were either

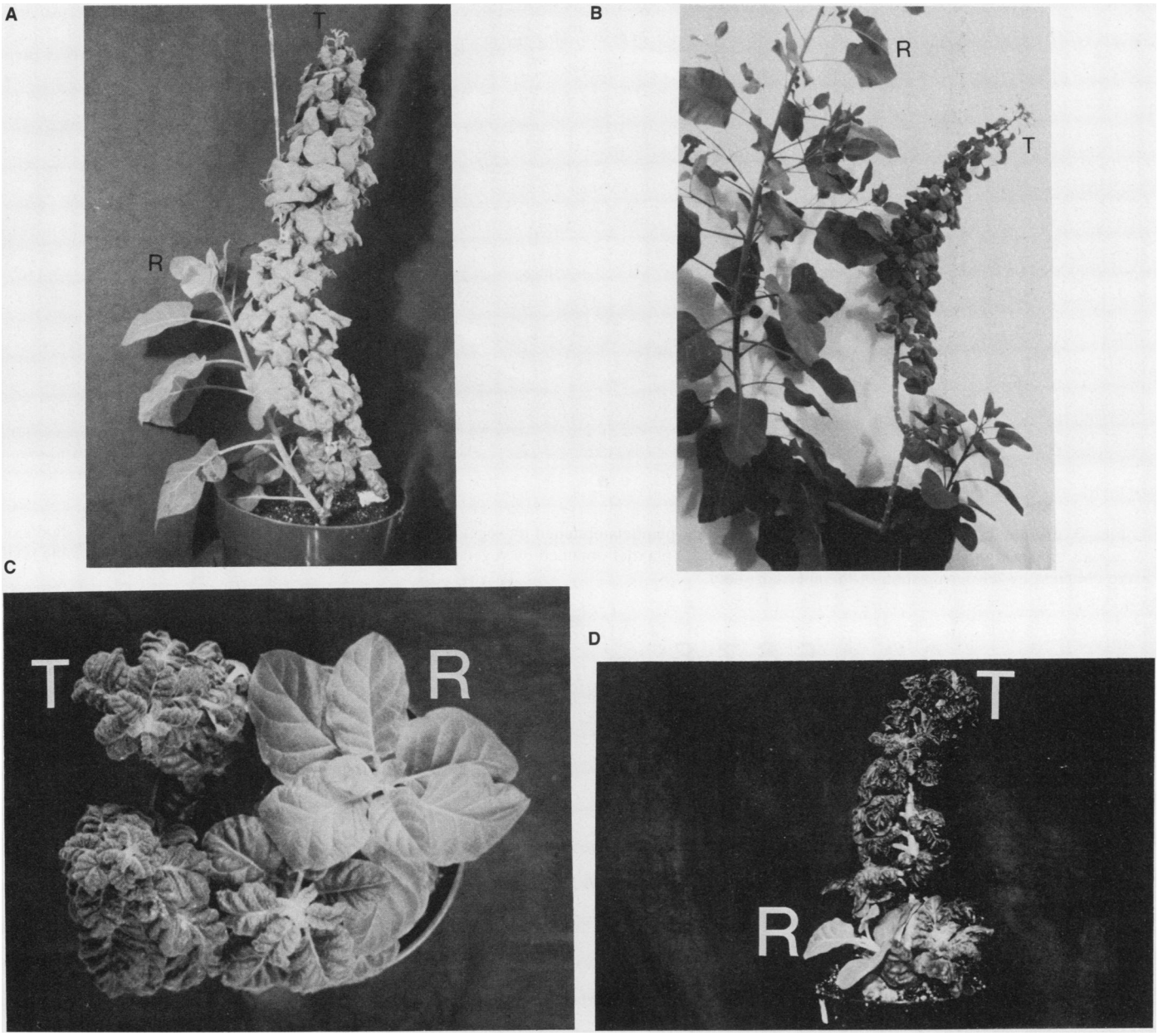


FIG. 2. Plants regenerated from the tissues transformed with the Ri plasmid and *rol*A locus. Stems of aseptically grown *N. glauca* were infected with *A. rhizogenes* R1000 and were grown axenically on $MS^-$. Roots produced by the tumors were regenerated into plants on the same medium. In case of *N. tabacum*, the leaf disc method described in "Materials and Methods" was used for obtaining transgenic plants. A), *N. glauca*, plant 1, showing normal looking revertant (R) shoot originating from the main aberrant shoot (T). Picture taken 2 months after the appearance of the revertant shoot. B), Same as in (A) except that the picture was taken 8 months after the appearance of the revertant shoot. Note the size of the revertant shoot. C), *N. tabacum* transformed with the Ri plasmid showing aberrant shoot (T) and the revertant shoot (R). Picture taken 2 months after the appearance of the revertant shoot. D), *N. tabacum* transformed with *rol*A locus alone showing aberrant shoot (T) and the revertant shoot (R). Picture taken 1 month after the appearance of the revertant shoot.

completely absent or at least a transcript of 0.8 kb was not detected in revertant shoots, indicating transcriptional inactivation of $T_L$-DNA genes in revertant shoots.

Inactivation of foreign DNA is not uncommon in plants (2, 11, 25). In a detailed study performed with primary tumors formed on *N. tabacum*, Van Lijebettens *et al.* (25) found that a significant proportion (15%) of transformed cells expressed Ti T-DNA hormone genes only transiently and subsequently became hormone dependent. In the same study they observed that about 46% of the cells within primary tumors, grown hormone independently, did not express or eventually stopped expressing the T-DNA genes. Although the loss of hormone autonomy was associated with the loss or rearrangement of T-DNA genes in many instances, in some of such cells the T-DNA did not undergo any noticeable change during the loss of hormone autonomy. In a few such cases investigated in that study T-DNA hypermethylation was thought to be associated with the inactivation of the genes. DNA hypermethylation has been also shown to be associated with transcriptional inactivation of the *tmr* gene of the nopaline type Ti plasmid in transgenic *N. tabacum* (2) and of nopaline synthase gene in flax (11). We have also found that the $T_L$-DNA region is hypermethylated in revertant shoots and demethylation results in reactivation of the $T_L$-DNA genes (VP Sinkar, unpublished data). So far, we have not observed de-

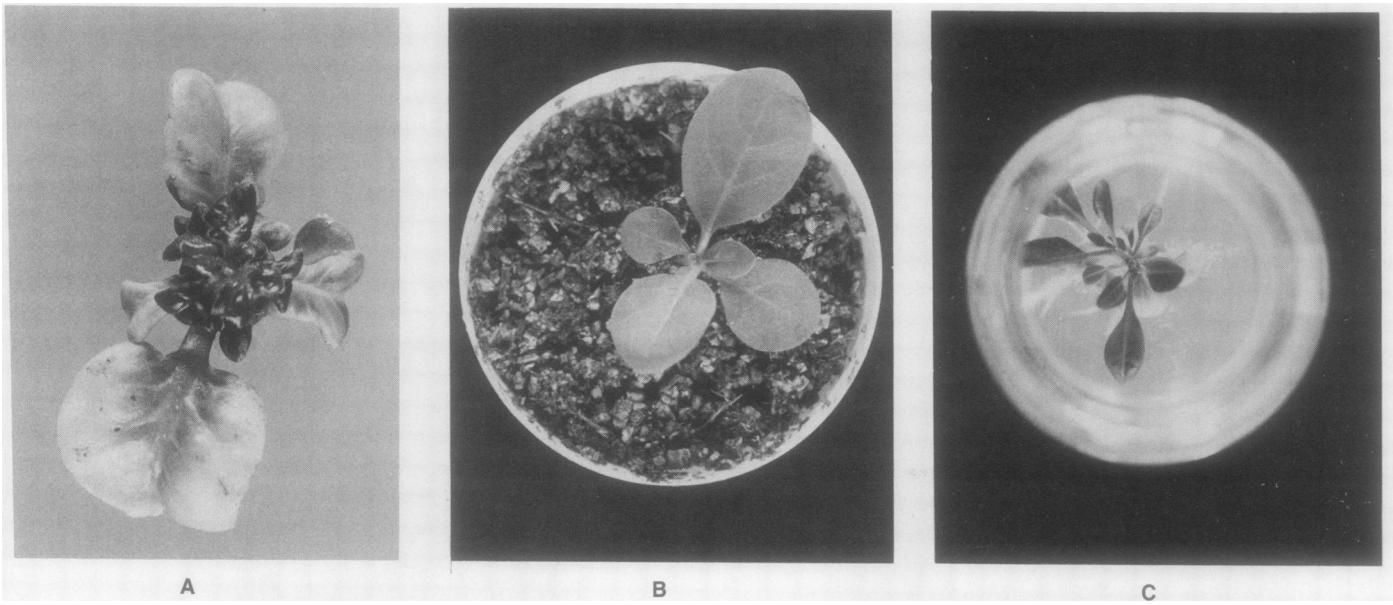


FIG. 3. Heritability of the revertant phenotype. Typical plantlets obtained by vegetative and sexual reproduction from the revertant shoots of *N. glauca* are shown in C and B, respectively. A, A typical plantlet obtained by vegetative reproduction from the aberrant shoot. No viable seed were obtained by self-fertilization of the aberrant shoot.

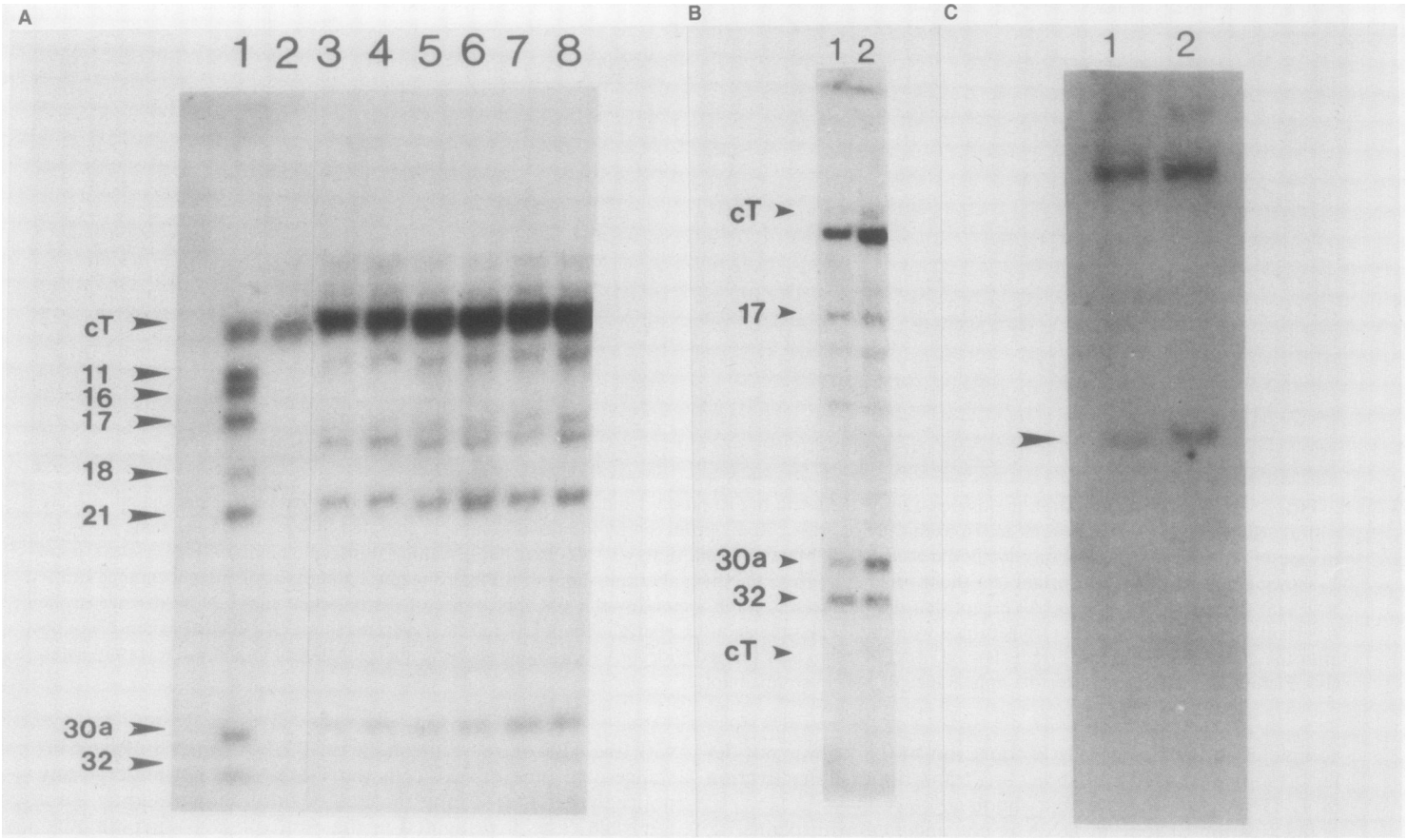


FIG. 4. T-DNA analysis of the transgenic plants. DNA isolated from the revertant and aberrant shoots of *N. glauca* (A), of *N. tabacum* transformed with the Ri plasmid (B), and of *N. tabacum* transformed with *rol*A locus (C) was digested with *Hin*dIII, separated electrophoretically on agarose gel, transferred to nitrocellulose membranes and was probed with pFW 302, a cosmid clone containing Ri $T_L$-DNA (see Table I). Key: (A) 1, one copy reconstruction; 2, untransformed *N. glauca*; 3, revertant shoot of plant 1; 4, aberrant shoot of plant 1; 5, revertant shoot of plant 2; 6, aberrant shoot of plant 2; 7, revertant shoot of plant 3; 8, aberrant shoot of plant 3, cT DNA, endogenous T-DNA. (B) 1, revertant shoot of *N. tabacum* transformed with Ri plasmid; 2, aberrant shoot of the same plant. (C) 1, revertant shoot of *N. tabacum* transformed with *rol*A; 2, aberrant shoot of the same plant. A 3.2 kb *Hin*dIII fragment indicative of the presence of *rol*A gene in plants is shown by an arrow.

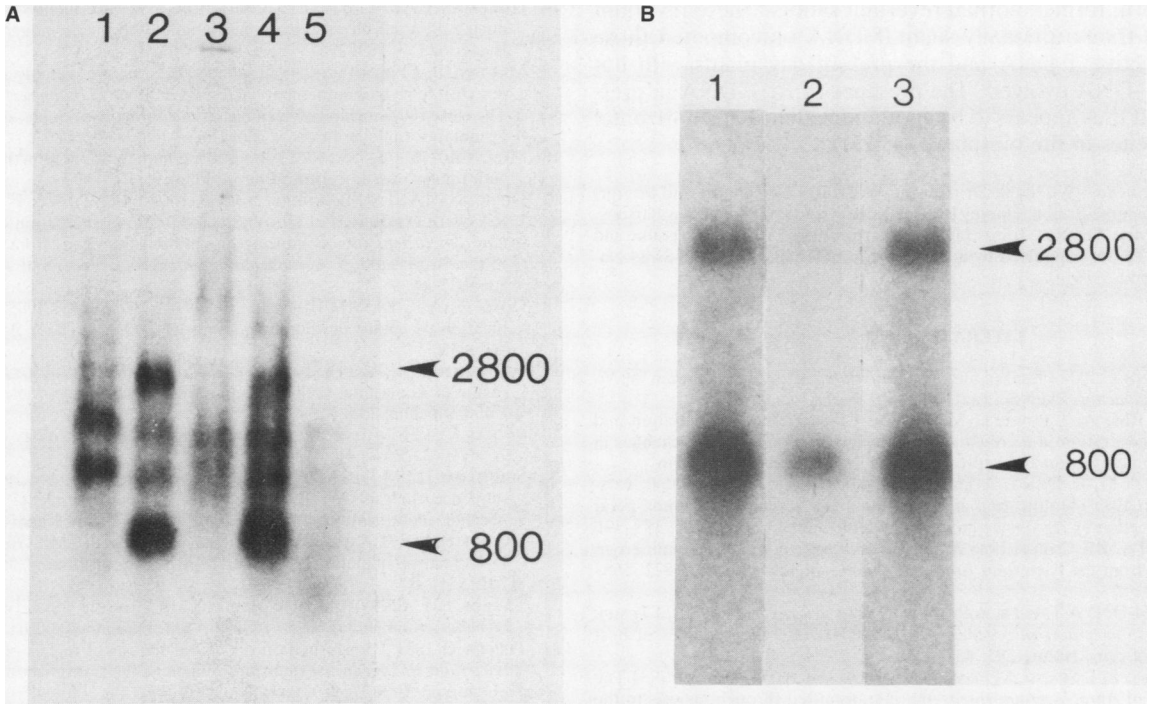


FIG. 5. Northern analysis of transgenic plants. Total RNA obtained from revertant and aberrant shoots of *N. glauca* plants 1, 2, and 3 was fractionated oligo-dT cellulose column to enrich for poly($A^+$) RNA. Three microgram of the poly($A^+$) RNA was fractionated on formaldehyde-agarose gel, was blotted onto nitrocellulose paper and was hybridized with various $T_L$-DNA probes. A, Hybridization results obtained with plants 1 and 2; B, those obtained with the aberrant shoot of plant 3. Key: (A) 1, revertant shoot of plant 1; 2, aberrant shoot of plant 1; 3, revertant shoot of plant 2; 4, aberrant shoot of plant 2; 5, untransformed *N. glauca*. Probe used for the analysis was pFW 302. (B) 1, aberrant shoot of plant 3 probed with pFW 302; 2, probed with Ri *Hin*dIII fragment 21; 3, probed with *Hin*dIII fragment 30a (note: no $T_L$-DNA homologous transcripts were detected in the revertant shoots of this plant).

tectable rearrangement of T-DNA in any revertant shoots; however, it is possible that similar to deletions observed in the T-DNA of transformed calli, T-DNA of revertant shoots would undergo changes over several generations as probably happened with endogenous T-DNA of some plant species (see the last paragraph of "Discussion").

The high frequency of reversion in transgenic plants may imply a presence of a cellular process directed toward silencing foreign genes in plants. On the other hand, plants have highly methylated DNA compared to that of animals (total of 30% cytosine residues are methylated in plants as opposed to about 1–5% in animal DNA; 10) and foreign DNA, assuming random integration, would frequently integrate into inactive chromatin structures. Such DNA would remain silent unless strong selection pressure favors its expression. This process, though random, may also appear like a process directed against expression of foreign genes since it would lead to frequent inactivation of foreign genes. In case of *A. rhizogenes* transformed plants, combination of the ability of plants to silence foreign genes and selection pressure favoring nonfunctional $T_L$-DNA genes increase the possibility of reversion. Axillary buds usually show reverted shoots probably because after derepression of the growth of axillary bud meristematic cells, the cells with inactive $T_L$-DNA genes grow rapidly and constitute the majority of the population in new shoots and hence the normal appearance. We regenerated plants from leaves, from axillary buds, and from internode regions of aberrant shoots of *N. glauca* (about 10 months old) and found that leaves and internodes produced aberrant plantlets or teratomas whereas axillary buds produced normal plants. In very young plants (less than 2 months old) all parts produced aberrant shoots while in very old plants (more than 18 months old) axillary buds, and often internodes also, produced normal looking shoots. Therefore, it seems likely that silencing of $T_L$-DNA genes, at least in *N. glauca*, is a function of the age of the plant.

Some species of the genus *Nicotiana* contain DNA sequences homologous to the Ri $T_L$-DNA (9, 27, 28). Possible explanations for the presence of the homologous sequences between *A. rhizogenes* and plants are: (a) that *A. rhizogenes* has in its evolutionary history captured these genes from plants, which it now reinserts into the plant genome, analogous to the acquisition of host oncogenes by RNA tumor viruses, (b) that these sequences represent essential genes in plants and bacteria and hence are conserved in both; and (c) that the endogenous T-DNA sequences are the result of a past infection by *Agrobacterium* of plants. The distribution of Ri-$T_L$-DNA homologous sequences is limited only to two subgenera of *Nicotiana* and not all members of these groups have it. In at least one species, *N. tabacum*, the large central region corresponding to bacterial *rol*B and *rol*C is absent. Thus, the Ri-TL-DNA sequence shows a scattered distribution and apparently is not present in many plants. The scattered distribution argues against the hypothesis that the homologous T-DNA sequences are essential genes which are involved in plant growth. These sequences are not found in other bacteria including *A. tumefaciens*. Furthermore, *A. rhizogenes* strains lacking these genes are still virulent suggesting their nonessential role in agrobacteria. Therefore, the possibility that these sequences in plants represent past infection by *A. rhizogenes* seems to be more plausible. Our observations suggest that during the past, an infection of *Nicotiana* or a progenitor of the *Nicotiana* species by *A. rhizogenes* resulted in generation of aberrant plants

which, in turn, formed normal revertant shoots. These revertant shoots with transcriptionally silent T-DNA outcompeted those harboring active T-DNA and the present day plants with homologous T-DNA evolved. The presence of Ri T-DNA in present day plant thus appears to be another incidence of a horizontal spread of genes in the biosphere (4, 15).

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