Reversion of Aberrant Plants Transformed with Agrobacterium rhizogenes Is Associated with the Transcriptional Inactivation of the T \(_L\)-DNA Genes

Received for publication July 30, 1987 and in revised form October 19, 1987

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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\(^a\) 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 intensively complemented (14, 17, 30). The tms1 gene product, tryptophan monooxygenase, converts tryptophan to indoleacetaldehyde which is then hydrolyzed by the gene product of tms2, indoleacetaldehyde hydrolase, to IAA (19). The T \(_L\)-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 Kalanche \(_\text{e} \) diabrotica leaves (3, 26, 30). Similarly, four T \(_R\)-DNA loci (rolA, rolB, rolC, and rolD) have been identified by transposon mutagenesis that affect tumor phenotype on K. diabrotica 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 hypersyly. The transformed phenotype has been associated with the T \(_L\)-DNA (22), and particularly with the rolA 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...
Nicotiana tabacum plants transformed with the Ri plasmid or with rolA locus. We present evidence in this communication that the phenomenon of reversion is associated with the transcriptional inactivation of the T-DNA genes and not with detectable rearrangement of the T-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 μg/ml kanamycin (Sigma), 100 μg/ml carbenicillin (Geopen, Pfizer Ltd), and 100 μg/ml gentamycin (Sigma). E. coli strains were maintained on L-agar supplemented when appropriate with 100 μg/ml kanamycin, 100 μg/ml carbenicillin, 10 μg/ml tetracyclin (Sigma) or 50 μg/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 μg/ml). These roots frequently regenerated into plants.

To obtain transgenic plants containing rolA locus, N. tabacum leaf discs were infected with A. tumefaciens LBA 4404 (a helper strain which provides the vir functions in trans: 12) containing rolA locus (pFP3, VP Sinkar, unpublished data). The leaf discs were grown on MS 104 growth medium (13) containing kanamycin (100 μg/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 procedure 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 NaHPO4 (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 32P were denatured and added to the same mix to obtain 1 × 106 cpm/ml. Hybridization was carried out for 16 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-

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

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
<th>Relevant Phenotype and Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td><strong>A. rhizogenes</strong></td>
<td></td>
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<tr>
<td>R1000 (pRiA4b)</td>
<td>onc+</td>
<td>Derivative of A4T (30)</td>
</tr>
<tr>
<td>R1500</td>
<td>onc+, CarbR, plant KanR in H 21</td>
<td>Derivative of pRiA4b (VP Sinkar, unpublished data)</td>
</tr>
<tr>
<td><strong>A. tumefaciens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBA 4404</td>
<td>onc-, SpecR</td>
<td>Derivative of pTiAch5 (12)</td>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>thr, leu, thi, pro</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pFW 302</td>
<td>TeR, RK2rep, IncP1</td>
<td>H-11 to H-18 of pRiA4b in pVK 102 (30)</td>
</tr>
<tr>
<td>pFP 3</td>
<td>TeR</td>
<td>rolA locus into pGA 472, a Ti plasmid derived vector for plant transformation (VP Sinkar, unpublished data)</td>
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tionated on formaldehyde agarose gel and was blotted 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-DNA or rolA 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 axial 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 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 acetic acid) and a cytokinin (6-benzylamino purine) 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 F1 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 hyperstam. 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 T1-DNA content by southern hybridization. DNA isolated from these shoots was digested with various cysteine methylation insensitive restriction enzymes and was probed with a cosmids clone containing the entire T1-DNA. The same T1-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 T1-DNA sequences (data are shown for HindIII digestion, Fig. 3A). All internal T-DNA fragments were detected in the anomalous fragment of 17 kb 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 HindIII fragment 21 and the other with rolA 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. HindIII 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 HindIII fragment of 3.2 kb indicative of the presence of rolA locus, which comprises part of the rolA locus and vector sequences, is present in both aberrant and revertant shoots of rolA containing N. tabacum (Fig. 4C). DNA obtained from the Ri and rolA 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 HindIII).

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. DNA isolated from leaves of both revertant and aberrant shoots was size-fractionated on formaldehyde-agarose gel, blotted onto nitrocellulose, and probed with T1-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 T1-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 T1-DNA while the aberrant shoot of the plant 3 expressed only two transcripts (0.8 and 2.8 kb; Fig. 5B) homologous to the T1-DNA. The revertant shoots of the plants 1 and 2 still expressed 1.2 and 1.6 kb transcripts homologous to the T1-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 HindIII fragments 21 and 30a of the T1-DNA (data shown for plant 3, Fig. 5B; see Fig. 1 for the restriction map of the T1-DNA). The sequence and genetic data indicate that the transcript emerging from the rolA locus would hybridize with both these fragments (33, 49), therefore it seems that the transcripts (at least the 0.8 kb) represent the rolA locus. Its association with the aberrant phenotype is consistent with our observations that the rolA locus controls the aberrant phenotype (VP Sinkar, unpublished data). We also performed T1-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 T1-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 T1-DNA and no rearrangement or deletions were noticed. However, T1-DNA specific transcripts were either
TRANSCRIPTIONAL INACTIVATION OF THE Ri T1-DNA

completely absent or at least a transcript of 0.8 kb was not detected in revertant shoots, indicating transcriptional inactivation of T1-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 T1-DNA region is hypermethylated in revertant shoots and demethylation results in reactivation of the T1-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 rolA locus (C) was digested with HindIII, separated electrophoretically on agarose gel, transferred to nitrocellulose membranes and was probed with pFW 302, a cosmid clone containing Ri T-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 rolA; 2, aberrant shoot of the same plant. A 3.2 kb HindIII fragment indicative of the presence of rolA gene in plants is shown by an arrow.
than 2 from internodes of N. of axillary buds, produced aberrant shoots while in very old plants (more than 18 months old) axillary buds, and internodes also produced normal looking shoots. Therefore, it seems likely that silencing of T\textsubscript{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\textsubscript{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\textsubscript{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 rolB and rolC 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).

Acknowledgements—We thank Dr. Eugene W. Nester for support and encouragement, and George Bolton, Harvey Bradshaw, Roy Kanemoto, Thomas Parsons, Ann Powell, Mir Sheller, Srinivasan and Martin Yanofsky for discussion and suggestion. F. P. was supported by a postdoctoral fellowship from the Swiss National Foundation.

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