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# T-DNA analysis of plants regenerated from hairy root tumors

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**Summary.** Plants regenerated from hairy root tumors induced on *Nicotiana glauca* and *Nicotiana tabacum* by *Agrobacterium rhizogenes* strain A4 were examined for the presence of T-DNA. Regenerated *N. tabacum* plants contained intact copies of both TL-DNA and TR-DNA. However, plants regenerated from *N. glauca* tumors did not contain the TR-DNA region corresponding to the *tms* (auxin synthesis) genes. Some of the regenerants exhibited an abnormal phenotype which is characterized by severe leaf wrinkling. This phenotype is correlated with the presence of TL-DNA, but not TR-DNA.

## Introduction

Several species of Agrobacteria promote tumor formation on dicotyledonous plants. *Agrobacterium rhizogenes*, the causative agent of hairy root disease, is characterized by its ability to cause root proliferation on susceptible hosts, while *Agrobacterium tumefaciens*, the causative agent of crown gall disease, causes the formation of either unorganized or teratomous tumors. Virulence in both of these species is dependent of large Ti (tumor-inducing) or Ri (root-inducing) plasmids, a portion of which is transferred and stably integrated into the host plant DNA (for a review, see Bevan and Chilton 1982; Nester et al. 1984). In contrast to tumors induced by *A. tumefaciens*, tumors induced by *A. rhizogenes* frequently regenerate to intact plants. The molecular basis of this reversion is unknown.

Recent studies on tumors induced by *A. rhizogenes* strain A4 on *Nicotiana glauca* have shown that two segments of Ri plasmid DNA, termed TL-DNA and TR-DNA, are transferred into host plant cells (White et al. 1985). Two loci located on the TR-DNA have been identified as *tms* auxin biosynthesis genes on the basis of homology to, and complementation of, the *tms* genes of *A. tumefaciens* (Huffman et al. 1984; White et al. 1985). Genetic evidence suggests that expression of the *A. rhizogenes tms* genes is the primary determinant of tumorigenesis on most host plants (White et al. 1985). In addition, four genetic loci have been identified on the TL-DNA and designated *rol* (rooting locus) genes for their effects on virulence and tumor morphology. One of these genes, *rol* B, is required for virulence on *Kalanchoe* leaves (White et al. 1982). A map of the T-region of the virulence plasmid pRiA4b of strain A4 showing the location of these loci and the boundaries of the TL-DNA and TR-DNA segments identified in hairy root tumors is shown in Fig. 1.

In order to determine whether changes in the T-DNA were responsible for the reversion of tumor cells to intact plants, we determined the T-DNA complements of plants regenerated from hairy root tumor tissue. Plants regenerated from tumors on *Nicotiana tabacum* contained both of the T-DNA segments found in *N. glauca* unorganized tumors, while in *N. glauca* regenerants, the region of the TR-DNA on which the *tms* genes are located was excluded. Some of the revertants exhibited an abnormal phenotype which has been correlated with the presence of T-DNA (Tepfer 1984). This abnormal phenotype is associated with the presence of TL-DNA, and not TR-DNA.

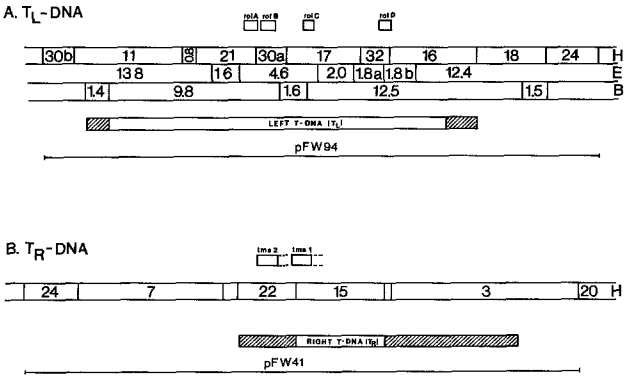


**Fig. 1.** Restriction fragment map of the pRiA4b T-DNA regions. The fragment designations for *Hin*dIII (H) are those assigned by Huffman et al. (1984). The fragment deisgnations for *Eco*RI (E) and *Bam*HI (B) are the fragment sizes in kilobases. The *bars* above the map indicate the regions of pRiA4b that are present in the *N. glauca* tumor lines (White et al. 1985). The *crosshatched portions* of the bars indicate the approximate ends of the T-DNA regions. The *boxes* above the map indicate the positions of morphogenic loci identified by White et al. (1985). The cosmid clones used in the hybridization experiments are indicated by bars at the bottom of the figure

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## Materials and methods

*Tumor and plant lines.* Tumors were initiated by stem inoculation of sterile *N. glauca* or *N. tabacum* cv. Xanthi plants with wild-type *A. rhizogenes* A4 (Moore et al. 1979) or a transconjugant strain (pRiA4b in an *A. tumefaciens* C58 chromosomal background; White and Nester 1980). Roots obtained from explanted tumors were cultured on MS media without hormones (Murashige and Skoog 1962) with or without 0.6% phytagar (Gibco, New York). Plantlets were obtained from roots either by spontaneous shooting or following treatment with 1 mg/l kinetin, and were subsequently grown in sterile culture or in soil. Tissue and plant lines are identified by tumor, root, plant (Ro), and progeny ($R_1$) numbers. For example, A4-4a:2:11:1 would be progeny plant 1 from parent plant 11 regenerated from root 2 of tumor line A4-4a. Tumor lines beginning with A4 were initiated with wild-type strain A4, tumor lines beginning with G (*N. glauca*) or X (*N. tabacum* cv. Xanthi) were initiated with the transconjugant strain.

*DNA isolation.* DNA was isolated by a modification of the procedure of Murray and Thompson (1980) as described by Amasino et al. (1984). Southern blotting and DNA hybridization conditions were also as described by Amasino et al. (1984).

*One copy reconstruction.* The ratio of the molecular weights of *N. glauca* (diploid $4.16 \times 10^{12}$ d; Galbraith 1983) and pRiA4b ($1.6 \times 10^8$ d; White and Nester 1980) is $2.60 \times 10^4$; therefore 385 pg of pRiA46 DNA were added to 10 µg of *N. glauca* DNA for a one copy reconstruction mixture. For *N. tabacum* one copy reconstructions 275 pg of pRiA4b DNA were added to 10 µg of plant DNA.

*Probe DNA.* The isolation and characterization of pFW94 and pFW41 were described by Huffman et al. (1984). Single restriction fragment clones were obtained and plasmid DNA was isolated as described by White et al. (1985).

## Results

### *T-DNA characterization*

Of 15 root lines derived from four independent *N. glauca* tumor explants, four contained detectable amounts of T-DNA: A4-4a root 2, A4-4a root 3, A4-4a root 4, and G1 root 10. Hybridization of labeled probes encompassing the left and right T-regions of pRiA4b to Southern blots of these root lines indicated that A4-4a root 2, A4-4a root 3, and A4-4a root 4 all contain fragments which comigrate with *Hin*dIII fragments 21, 30a 17, and 32, suggesting that these lines contain complete copies of TL-DNA (Fig. 2). The similarity of the T-DNA patterns of root lines A4-4a root 3 and A4-4a root 4 suggests that they may have originated from the same transformation event. Fragments which hybridized to the TR-DNA probe were detected only in A4-4a root 2 and G1 root 10 DNA. The fragments observed were subsequently shown to hybridize exclusively to *Hin*dIII fragment 3 (data not shown), indicating that the TR-DNA region corresponding to the *tms* genes is not present in these lines.

In order to determine whether similar T-DNA patterns were present in *N. tabacum* regenerants, DNA from regen-

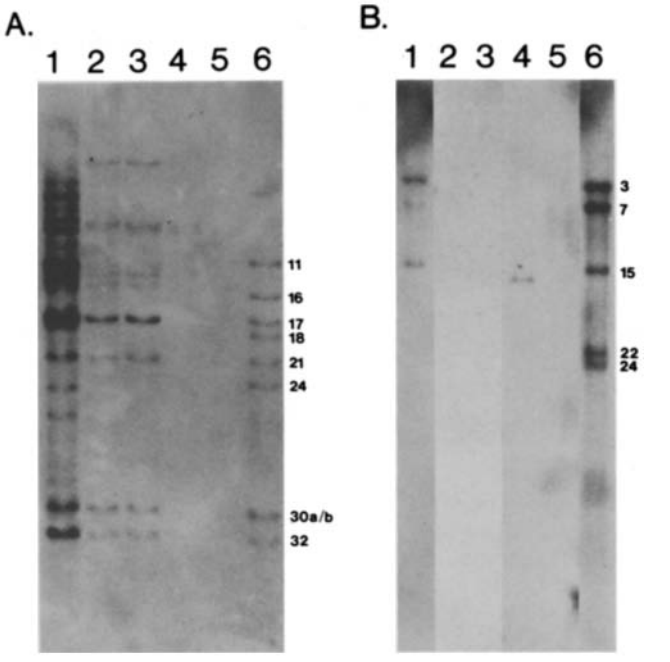


**Fig. 2A, B.** T-DNA analysis of *N. glauca* root lines. *Hin*dIII-digested root DNA was fractionated, blotted , and hybridized to $^{32}$P-labelled pFW94 (**A**) and pFW41 (**B**). Lane (1) contains A4-4a:2 DNA, lane (2) A4-4a:3, lane (3) A4-4a:4, lane (4) G1:10. Lane (5) is an untransformed *N. glauca* DNA control. Lane 6 is a 1 copy reconstruction. *Hin*dIII restriction fragment designations in the 1 copy reconstruction are given to the right of the autoradiograms

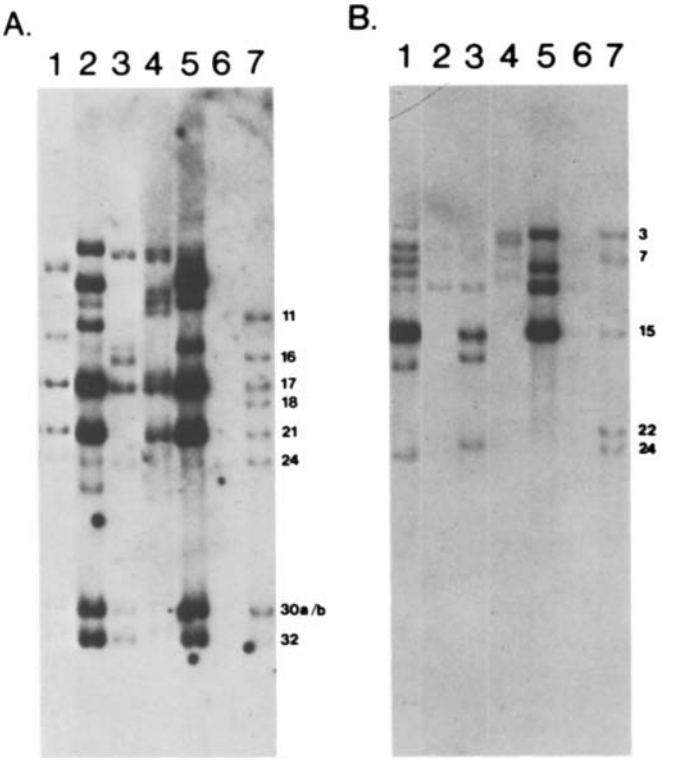


**Fig. 3A, B.** T-DNA analysis of $R_0$ *N. tabacum* regenerants. *Hin*dIII-digested DNA was fractionated, blotted, and hybridized to $^{32}$P-labelled pFW94 (**A**) and pFW41 (**B**). Samples are as follows: Lane (1) Xa:1:1, lane (2) Xb:2:1, lane (3) Xd:1:1, lane (4) Xf:3:1, lane (5) Xg:2:1, lane (6) normal *N. tabacum*, lane (7) 1 copy reconstruction. *Hin*dIII fragment designations in the 1 copy reconstruction are given to the right of autoradiogram


**Fig. 4.** Comparison of normal and abnormal *N. glauca* regenerant progeny. Plants shown are examples of normal and abnormal progeny of A4-4a:2:4, obtained by selfing

erants of five independent tumor lines was hybridized to TL-DNA and TR-DNA probes. The results are shown in Fig. 3. Regenerants Xa:1:1, Xb:2:1, Xf:3:1 and Xg:2:1 contained fragments which comigrated with *Hin*dIII fragments 21, 30a, 17, and 32 from the TL-DNA region. Regenerant Xd:1:1 contained fragments H-30a, H-17 and H-32 but did not contain H-21 as an intact fragment. Regenerants Xa:1:1, Xd:1:1 and Xg:2:1 also contained fragments which comigrated with intact *Hin*dIII fragment 15 from the TR-DNA region. These results indicate that *N. tabacum* regenerants can possess T-DNA complements similar to those of *N. glauca* tumor callus.

*Correlation of abnormal phenotype with TL-DNA*

Plants regenerated from the transformed *N. glauca* root line A4-4a: root 2 exhibited the abnormal regenerant phenotype (Fig. 4). In order to determine whether this phenotype was associated with TL-DNA, TR-DNA or both, progeny obtained by selfing two A4-4a: root 2 regenerants (A4-4a:2:plant 4 and A4-4a:2:plant 11) were analyzed to determine their T-DNA complements and scored for the presence of the abnormal phenotype.

The parent ($R_0$) plants have a T-DNA complement similar to that of the A4-4a root 2 line, exhibiting bands comigrating with the E-4.6, E-2.0, E-1.8 and E-1.6 *Eco*RI restriction fragments (Fig. 5). These bands were also detected in each of eight $R_1$ progeny; however, the distribution of the other bands in the sample varied indicating that segregation of T-DNA bands had occurred. Those bands which do not comigrate with bands in the one copy reconstruction are likely to contain plant/T-DNA junctions or rearranged T-DNA. Those bands which do comigrate with bands in the one copy reconstruction probably represent internal fragments of the T-DNA. $R_1$ progeny plants A4-4a:2:4:plant 6, A4-4a:2:4:plant 7, A4-4a:2:11:plant 1, and A4:2:11 plant 2 exhibited the abnormal phenotype, whereas A4-4a:2:4:plant 12, A4-4a:2:4:plant 14, A4-4a:2:11:plant 3, and A4-4a:2:11:plant 4 did not. Since all of the $R_0$ plants contain the same internal restriction fragments, the distribution of the additional bands was examined to see if a correlation between a particular band and the abnormal phenotype could be observed. One such band was observed and is indicated with an arrow in Fig. 5.

Segregation was also observed between those bands which hybridized to the TR-DNA probe. In this case, no correlation could be made between the presence of a particular band and the abnormal phenotype. Indeed, A4-

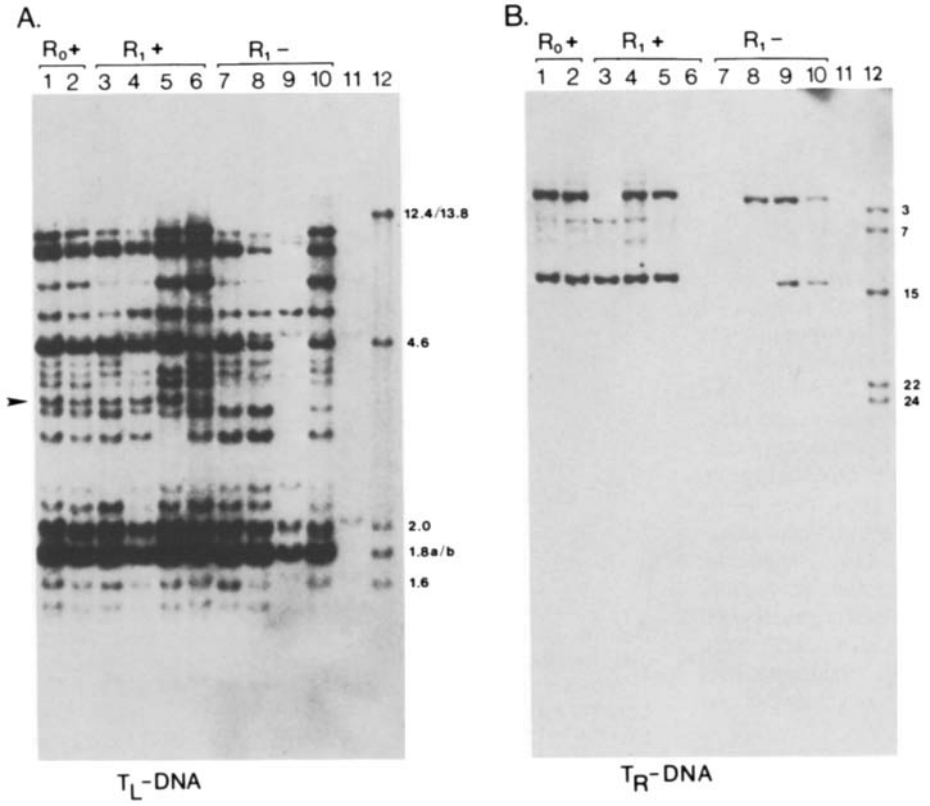


**Fig. 5A, B.** T-DNA analysis of $R_0$ and $R_1$ *N. glauca* regenerants. *Eco*RI (**A**) or *Hin*dIII (**B**) digested DNA isolated from *N. glauca* regenerants and progeny was fractionated, blotted, and hybridized to $^{32}$P-labelled pFW94 (**A**) or pFW41 (**B**). Samples are as follows: Lane (1) A4-4a:2:4, lane (2) A4-4a:2:11, lane (3) A4-4a:2:4:6, lane (4) A4-4a:2:4:7, lane (5) A4-4a:2:11:1, lane (6) A4-4a:2:11:2, lane (7) A4-4a:2:4:12, lane (8) A4-4a:2:4:14, lane (9) A4-4a:2:11:3, lane (10) A4-4a:2:11:4, lane (11) normal *N. glauca*, lane (12) 1 copy reconstruction. Bands observed with normal *N. glauca* DNA are cT-DNA (White et al. 1983). *Eco*RI and *Hin*dIII fragment designations in the 1 copy reconstruction are given to the right of the autoradiograms. (+) plants exhibit abnormal phenotype, (−) plants appear normal

4a:2:11:plant 2, which exhibited the abnormal phenotype, did not possess any sequences which hybridized to the TR-DNA probe. We conclude that the abnormal phenotype is correlated with the presence of TL-DNA and not TR-DNA. Similar analyses of transformed *N. tabacum* regenerants and their progeny have confirmed this conclusion (unpublished results).

## Discussion

In contrast to tumors induced by *A. tumefaciens*, tumors induced by *A. rhizogenes* revert to intact plants at a high frequency. We have compared the T-DNA of hairy root tumor callus and reverted roots and plants in order to identify molecular events which may have occurred during reversion. We have shown that plants regenerated from hairy root tumor tissue can contain two discrete segments of *A. rhizogenes* plasmid DNA. The specific segments detected were previously identified in DNA from unorganized *N. glauca* tumors (White et al. 1985). Tepfer (1984) determined that at least one T-DNA is maintained in *N. tabacum* plants regenerated from tumors incited with *A. rhizogenes* A4. Similar studies of plants regenerated from tumors initiated with the agropine type strain 1855 by Costantino et al. (1984) also detected one segment of T-DNA. The T-DNA identified in the previous studies corresponds to our TL-DNA. The possibility exists that TR-DNA sequences were also present in the plants analyzed by Tepfer (1984) and Costantino et al. (1984), since the T-DNA probes used in their studies did not extend into the TR-DNA region.

Since the *tms* genes of *A. rhizogenes* A4 are a prime determinant of virulence on all host plants tested, we compared the fate of these genes in two different plant species during regeneration. Plants obtained from *N. tabacum* tumors contained *tms* genes in multiple copies. On the other hand, we were not able to detect *tms* sequences in roots and plants regenerated from *N. glauca* tumors, although two of the regenerants did contain other portions of the TR-DNA. This difference in T-DNA complement may relate to the different tumor morphologies observed on these host species. Tumors on *N. glauca* consist primarily of unorganized callus tissue, whereas tumors on *N. tabacum* are largely composed of roots. This difference persists when the tumors are placed in sterile culture, suggesting that these two species respond differently to the hormone imbalance created by the T-DNA in the transformed cells. The auxin imbalance produced by the *tms* genes in transformed cells of *N. tabacum* may be insufficient to prevent differentiation of transformed cells into roots and intact plants, whereas in *N. glauca* cells *tms* gene function blocks differentiation and confers hormone autonomy on the transformed cells. If this is the case, only those *N. glauca* cells in which the *tms* genes are absent or inactive would be capable of regeneration into intact plants. Northern blot analysis of RNA from *N. tabacum* regenerants indicates that the *tms* genes in these plants are expressed at a low level relative to *tms* genes in *N. glauca* tumor calli, or are silent (B. Taylor, unpublished results). Selection against auxin gene expression may therefore exist during tobacco regeneration, however the stringency of the selection may be less than that required for *N. glauca* regeneration.

Many of the plants which regenerate from hairy root tumor tissue exhibit a specific abnormal phenotype, characterized by wrinkled leaves, decreased apical dominance, and increased adventitious rooting (Ackermann 1977; Tepfer 1984). The presence of this phenotype in the progeny of revertant plants was correlated with the presence of TL-DNA, but not TR-DNA. White et al. (1985) identified four loci on the TL-DNA which have effects on tumor morphology on *Kalanchoe* leaves. One or more of these loci appear to have a cytokinin-like function, based on the ability of the *A. rhizogenes* TL-DNA to complement mutations in the *A. tumefaciens tmr* gene. None of the TL-DNA morphogenic loci exhibit detectable homology to *A. tumefaciens* T-DNA (Huffman et al. 1984). Elucidation of the functions of these genes and their role in tumor formation and the abnormal regenerant phenotype should provide additional insight into the mechanisms of hairy root pathogenesis and normal root development.

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