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Molecular biology of Ri-plasmid—A review

VILAS P. SINKAR, FRANK F. WHITE*, and MILTON P. GORDON
Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA
*Department of Plant Pathology, Kansas State University, Manhattan, Kansas 66506, USA

Abstract. *Agrobacterium rhizogenes* transfers a segment of its plasmid to the plant genome. The transferred DNA contains genes which are involved in the synthesis of plant hormones. These genes express in the plant cell and give rise to rooty-tumors at the infection site. Transgenic plants can be readily regenerated from the rooty-tumors and the transferred DNA is transmitted to progeny plants. High regeneration potential and sustained maintenance of transferred DNA makes the bacterium a suitable vector for plant genetic engineering. DNA sequences homologous to the transferred DNA of *Agrobacterium rhizogenes* were detected in some untransformed plant species suggesting a past infection by *Agrobacterium rhizogenes* during evolution of some genera, notably Nicotiana.

Keywords. *Agrobacterium rhizogenes*; Ri-plasmid; endogenous T-DNA.

Introduction

Agrobacterium rhizogenes is a gram negative soil bacterium. It incites hairy root disease of many dicotyledonous plants (Brown, 1929; DeCleene and DeLey, 1981; Riker *et al*.,1930; Siegler, 1928). The ability of A. *rhizogenes* to incite hairy root disease is determined by a virulence plasmid (Chilton. *et al*.,1982; Moore *et al*.,1979; White and Nester, 1980) similar to that found in *Agrobacterium tumefaciens* which causes Crown gall tumors of plants. The virulence plasmid of *A*. *rhizogenes* is known as the Ri-plasmid to distinguish it from the tumor-inducing (Ti) plasmid. Extensive literature is available pertaining to the Ti-plasmid. Therefore, we will not describe the Ti-plasmid in detail except where necessary for comparison with the Ri–plasmids. For more information on the Ti-plasmid readers are referred to some recent review articles (Nester *et al*.,1984; Hille *et al*.,1984; Zambryski *et al*.,1983).

The Ri-plasmid shares extensive functional homology with the Ti–plasmid. The Ri–plasmid, like the latter, contains a distinct segment (s) of DNA which is transferred to plant genome during infection (Chilton *et al*.,1982; White *et al*.,1982; Willmitzer *et al*.,1982). The transfer of the DNA (T-DNA) to the plant genome is mediated by another segment on the plasmid known as the virulence (*vir*) region. The T-DNA confers on the plant cells the ability to grow in the absence of exogenous plant hormones. The T-DNA also confers on the transformed tissue the ability to produce modified amino acids (opines), which, in turn, are utilized only by the inciting bacteria as the carbon, nitrogen and energy source. The *Agrobacterium* species thus establish a unique ecological niche by genetically engineering the host plant—a highly sophisticated parasitism!

DNA sequences homologous to the T-DNA of Ri –plasmids were reported in some untransformed plant species (Spano *et al*., 1982; White *et al*.,1982, 1983; Tepfer, 1984). The presence of these homologous sequences suggest the possibility that such

Abbreviations used: Ri, root-inducing; T_L–DNA, left T–DNA; T_R–DNA, right T–DNA; T_i, Tumour inducing; *vir*, virulence.

bacterially acquired sequences play an important role in the evolution of some plant species. Whether, in fact, these sequences contribute significantly to evolution or simply represent 'genetic scars' remains to be seen.

Ri -plasmid

Large plasmids were shown to be present in strains of *A. rhizogenes* (Schell *et al.*, 1976; Currier and Nester, 1976; White and Nester, 1980a). These strains are known to produce at least two classes of opines. One such class is represented by opines of agropine group, and the other class being the agrocinopine group. All strains of *A. rhizogenes* are known to produce agrocinopine and all or a few opines of the agropine group. The strains which produce ally the agropine-type opines (agropine, mannopine, agropinic acid and mannopinic acid) are known as the agropine-type strains, whereas the strains which produce all agropine-type opines excluding agropine are known as the mannopine-type strains (Petit *et al.*,1983; Tempe *et al.*, 1984; Tepfer and Tempe, 1981; White *et al.*,1982; Willmitzer *et al.*,1982).

Two T-DNA regions have been identified in agropine Ri-plasmids (see figure 1). The two tDNAs are separated from each other by about 15 Kb of non-transferred DNA. The right T–DNA (T_R) contains genes homologous to the T–DNA from Ti–plasmids (Riseuleo *et al.*, 1982; Willmitzer *et al.*, 1982; Huffman *et al.*, 1984; Jouanin, 1984). Most important among these are the genes homologous to the *tms1* and *tms2* of the Ti–plasmid. These genes are involved in auxin biosynthesis in *A. tumefaciens* (Inze *et al.*, 1984; Schröder *et al.*, 1984; Thomashow *et al.*, 1984; Thomashow *et al.*,

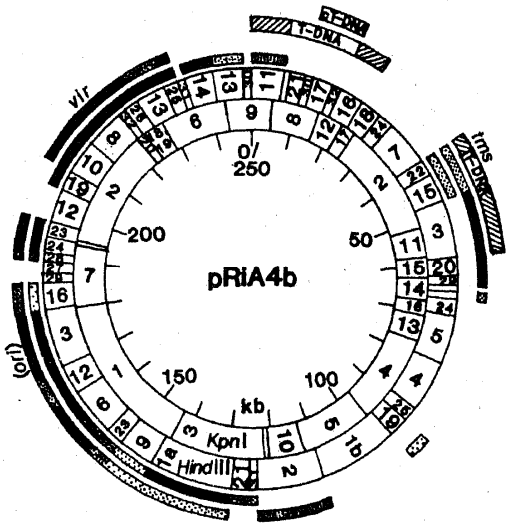


Figure 1. Restriction map of an agropine type Ri –plasmid (pRiA4b). Data taken from Huffman *et al.* (1984). Homology to the octopine type Ti–plasmid (pTiA6) is indicated by outer most thin bars while homology to the nopaline type Ti–plasmid (pTiT37) is indicated by the inner bars. Dark regions represent stronger hybridization signal.

Vir, virulence region; tms, auxin genes; ori, origin of replication; cT DNA, region of DNA homology between pRiA4b and untransformed *N. glauca*.

1986). Mutagenesis of this region in the Ri–plasmid is shown to result in the loss or attenuation of virulence (White *et al*., 1985). Hybridization experiments also suggested the presence of the genes involved in agropine biosynthesis (*ags*) in the T_R-DNA region although the exact number of genes involved in agropine biosynthesis is not known yet (Huffman *et al*.,1984; Lahners *et al*.,1984; Willmitzer *et al*.,1982).

Nicotiana glauca tissues transformed with *A. rhizogenes* contain discrete m-RNA species derived from the T_R-DNA. The transcripts homologous to the Ri *tms* loci in such tissues were found to be of size similar to the transcripts derived from the *tms* region of Ti-plasmids (Taylor *et al*., 1985a; Willmitzer *et al*., 1983). Additional transcripts were also detected from such tissues and probably correspond to the *ags* region. Similar transcripts have also been reported from carrot plants regenerated from tissues infected with *A. rhizogenes* (De Paolis *et al*., 1985). However, the T_R-DNA transcripts are not detectable in regenerated *N. glauca* plants and are either absent or present at very low levels in regenerated *Nicotiana tabacum* plants (Taylor *et al*., 1985b; Durand-Tardif *et al*., 1985).

The left T-DNA (T_L) of agropine Ri-plasmid A4b is about 20 Kb in length but, unlike the T_R–DNA does not appear to be closely related to any other characterized Ti-plasmid (Huffman *et al*., 1984). Limited homology has been reported to the T–DNA of nopaline type Ti-plasmids, presumably to the region involved in the synthesis of agrocinopine (Huffman *et al*., 1984; Willmitzer *et al*.,1982). Transposon mutagenesis of the T_L-DNA has revealed the presence of at least four loci (*rolA*, B, C and D) which affect tumorigenesis in some plants (White *et al*., 1985; Estramareix *et al*., 1986). Mutations in *rolA* (*rol*A^-) results in the formation of long, straight roots giving the tumor a less compact appearance on *Kalanchoe diagremontiana* leaves (figure 2). The *rolB* mutation eliminates both callus or root formation at the wound site, while *rol*C^- and *rol*D^-mutations are more subtle (figure 2); *rol*C^- resulting in retardation of root growth and *rol*D^-in accentuation of callus growth giving rise to tumors resembling the Ti-plasmid infection. Transposon insertions between *rol*B and *rol*C showed weakened response on *K. diagremontiana* leaves implying the presence of another genetic locus (see figure 2D; White *et al*., 1985). However, the degree of the weakened response varied considerably among the mutants in this region and between inoculations. Since such results are possible if the insertions have affected expression of the adjacent loci, no *rol* locus has been assigned to this region.

The *rol* mutations present a rather intriguing picture of the T_L-DNA functions. White *et al.* (1985) observed apparent similarity between *tmr* and *rol*B^- mutations on *K. diagremontiana* leaves. Both mutants are unable to produce any callus or roots at the infection site on the leaves. Virulence of *tmr* mutants can be restored on the leaf by complementation with the Ri T_L-DNA (White *et al*., 1985). These observations suggest that the T_L-DNA is either involved in cytokinin biosynthesis or in altering cytokinin metabolism in plants. Current work in our laboratory indicates that the individual *rol* genes, including *rol* B *in trans*, are unable to restore complete virulence of *tmr* mutant strains on *Kalanchoë* leaf, thus indicating that more than one gene is involved in the synthesis or alteration of metabolism of cytokinin.

Slightom *et al.* (1986) sequenced 21,126 base-pairs of the T_L–DNA and found potential for 18 open reading frames. 27 consensus border recognition sequences homologous to those found in the Ti-plasmids which determine boundaries of the T-region were also detected throughout the T_L–DNA (Simpson *et al*., 1982; Yadav *et*

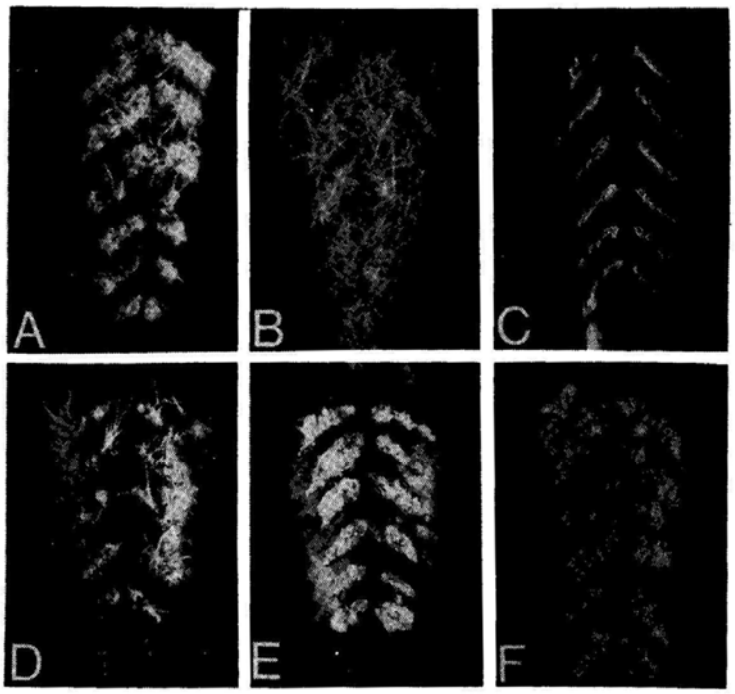


Figure 2. Effect of mutations of the T_L–DNA on tumor formation on Kalanchoë leafs. (A), R1000 (wild type); (B), R1022 (*rol*A$^-$); (C), R1023 (*rol*B$^-$); (D), R1020 (no *rol* locus assigned); (E), R1016 (*rol*C$^-$); (F), R1244 (*rol*D$^-$).
Pictures taken one month after infection (White *et al.,* 1985).

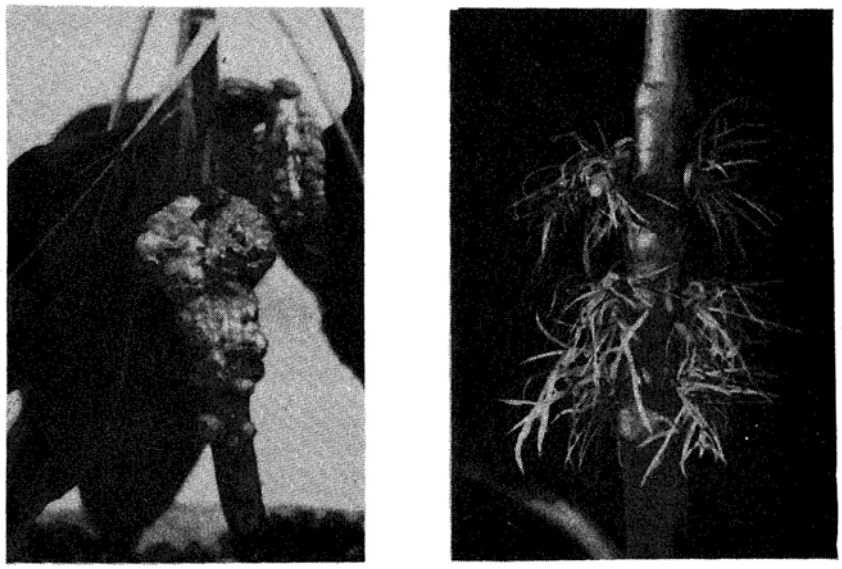
al., 1982; Zambryski *et al.,* 1982). The variability found in the boundaries of the T_L-DNA in different plants may occur due to the preferential use of different border recognition sequences during T–DNA transfer (Slightom *et al*., 1985; Tepfer, 1984; Taylor *et al*., 1985a; White *et al*., 1985). Durand-Tardif *et al.* (1985) and Taylor *et al.* (1985a) showed that the T_L–DNA is transcribed in both transformed tissue and regenerated plants. Although there are some differences in the two sets of data and all the individual transcripts are not found in every tissue or plant, careful inspection suggests that the transcripts roughly map to the open reading frames. Some of the anomalies could be explained due to turn-off and tissue-specific expression of some of the T_L–DNA genes.

Comparitively less data are available for the mannopine type Ri –plasmids. The best characterized mannopine type plasmid, that from strain 8196 contains only one T–DNA (Byrne *et al*., 1983). Hybridization data with agropine type plasmid (pRil855) and of pRi 8196 suggest that the Ri T_L–DNA including the *rol* loci are probably conserved (Spano *et al*., 1982). Since the genes for mannopine synthesis are located towards the right border of the T-DNA a possible fusion of the left and right T-DNA to obtain a single T-DNA in this strain is indicated (Lahners *et al*., 1984). The most striking difference between the agropine and mannopine type Ri –plasmids, however, seems to be the absence of *tms* genes in the latter. Whether non-homologous auxin biosynthetic genes are present on these plasmids is yet to be determined.

The physical structure and hybridization data strongly suggest a functional similarity between *vir* regions of the Ri- and Ti-plasmids (figure 1; White and Nester, 1980b; Hooykaas *et al*.,1984; Hoekema *et al*.,1984; Hoffman *et al*.,1984; Willmitzer *et al*.,1982). Hybridization data between pRiA4b and the two most studied Ti-plasmids, pTiA6 and pTiT37, suggest that all the known *vir* loci are present on the Ri- plasmid (Huffman *et al*., 1984; Unger *et al*., 1985). One exception to this finding is the lack of hybridization to *vir*E probe. *Vir* mutations in Ti-plasmid including *vir*E can, however, be complemented by the virulence region of the Ri–plasmid (Hooykaas *et al*., 1984; White, F.F., unpublished results). The reason for the lack of the homology to the *vir*E despite of functional complementation is yet unknown.

Ri–transformed plants

A. rhizogenes induced tumors have a distinct morphological phenotype on many plant species, notably the pronounced rooting, compared to tumors derived from strains containing Ti-plasmids (figure 3). Moreover, the Ri-plasmid transformation of plants does not appear to be inhibitory to normal differentiation as the incorporation of T–DNA of the Ti–plasmid containing the phytohormone synthesis genes. Plants can be readily regenerated from the callus or roots of Ri-transformed plant tissues (Ackermann, 1977; Chilton *et al*., 1982; David *et al*., 1984; Spano and Costantino, 1982; Tepfer, 1982, 1984; Taylor *et al*., 1985a), and the T–DNA can be



Figured 3. (A), Tumor formation by A. *tumefaciens*, A348 (octopine type plasmid), on *Helianthus* sp. (B), Root proliferation induced by A. rhizogenes A4b on the stem of *K. diagremontiana*.

subsequently transmitted to progeny plants (Costantino *et al*., 1984; Tepfer, 1984; Taylor *et al*., 1985a; Sinkar V. P., unpublished results). The analysis of T–DNA of the regenerated plants suggest that the incorporation of the *tms* genes is inhibitory to regeneration in some species. In such species, *e.g*., *N. glauca*, only those cells which either have lost the *tms* genes or the T_R–DNA or never received the T_R–DNA regenerate whole plants (Taylor *et al*., 1985b). On the other hand, the regeneration process in some plants such as *N. tabacum* appears to be less affected by the presence of the *tms* genes (Taylor *et al*., 1985a). In some instances, however, regenerated *N. tabacum* plants do not contain any T_R–DNA (Durand-Tardif *et al*., 1985; Sinkar V. P., unpublished results). As a general rule, it seems that severely reduced activity or inactivation of the *tms* loci is a prerequisite for plant regeneration from the Ri-plasmid transformed tissue.

Regenerated plants containing Ri-plasmid T– DNA often exhibit many phenotypic abnormalities, such as wrinkled leaves, shortened internode distances, loss of apical dominance, increased lateral root-formation and heterostyly (figure 4); Ackermann, 1977; Tepfer, 1984; Taylor *et al*., 1985b; Ooms *et al*., 1985; Sinkar V. P., unpublished results). In wrinkled *N. glauca*, the T_R–DNA pattern indicates that complete copies of either the *tms* loci or entire T_R–DNA sequences were absent (Taylor *et al*., 1985b; Sinkar V.P., unpublished results). Similar observations have also been made with *N. tabacum* and *Solanum tuberosum* (Durand-Tardif *et al*., 1985; Sinkar V. P., unpublished results; Ooms *et al*., 1985). However, all abnormal plants tested so far always showed the presence of the T_L–DNA. Thus, it seems that some gene or combination of genes in the T_L–DNA controls the aberrent phenotype associated with the regenerated plants.

Figure 4. N. tabacum regenerates from tissue culture. Plant A was regenerated from pRiA4b transformed leaf tissue of *N. tabacum*. Plant B represents normal *N. tabacum*.

Endogenous tDNA of plants

Initial characterization of T–DNA both with agropine and mannopine Ri-plasmids led to the observation of hybridization to some untransformed plant species. In the case of the agropine type Ri-plasmid, A4b, strong hybridization to *N. glauca* genome was observed with the T_L–DNA of the plasmid (White *et al*., 1982; White *et al*., 1983). The homologous plant sequences (about 12 Kb in size) were cloned and sequenced (Furner *et al*., 1986). Of these 12 Kb, about 7·5–8·0 Kb is the central T–DNA region and the remainder is in asymmetric inverted repeats (figure 5). Similar homologous sequences were also detected in other species of *Nicotiana*, *e.g*., *N. tabacum, N. octophora, N. tomentosiformis* and *N. benavedesii* (Furner *et al*., 1986). Various wild isolates of *N. glauca* obtained from different parts of the world also showed the presence of similar sequences, although restriction patterns differed (Furner *et al*., 1986).

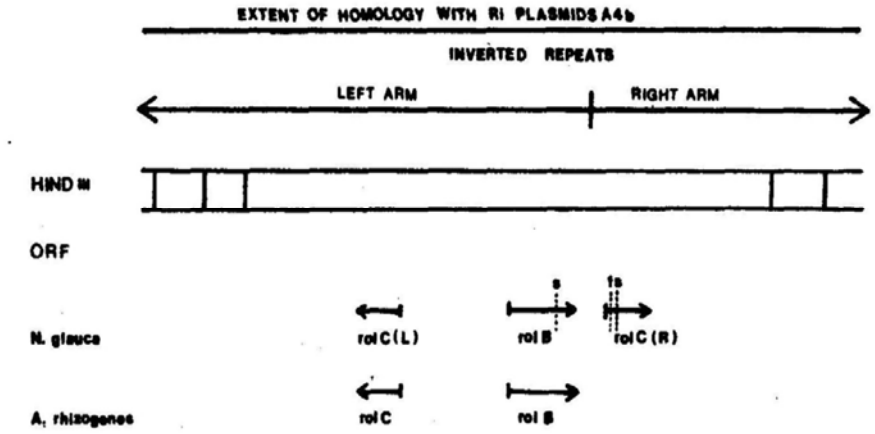


Figure 5. Organization of Ri T_L-DNA homologous sequence *N. glauca*. Hind III map of the homologous region is shown. Open reading frames deduced from DNA sequence data are depicted as arrows at the bottom. Corresponding open reading frames from the Ri-plasmid A4b are shown for comparison. Beginning of the inverted repeats is represented by a vertical bar. (Data taken from Furner *et al*., 1986).

(ORF), Open reading frame; (F), frameshift; (S), stop codon; (L), left; (R), right.

The region of homology between *N. glauca* and pRiA4b includes *rol*C, *rol*B and an undetermined region of *rol*D. DNA sequence analysis showed that the left plant copy of the *rol*C locus contains the complete open reading frame. The right copy of the plant *rol*C contains a frame shift and a termination codon near the start codon implying that this copy is not potentially functional. The open reading frame of the plant *rol*B can potentially encode a polypeptide that is truncated by 48 amino acids at the carboxyl terminus (see figure 5, Furner *et al*., 1986). These open reading frames share about 75% DNA and 84% amino acid homology with the corresponding regions of the Ri-plasmid.

Many questions are raised by the presence of endogenous T–DNA. The foremost among them are whether the endogenous T–DNA contains any active genes and, if so, whether the gene products have any function in the plant cell. No evidence of transcriptional activity of these genes has been observed yet indicating that these

genes are either silent or are expressed only transiently. Experiments are in progress to use the plant genes to complement Ri-plasmid mutations in *rol*B and *rol*C. These experiments including analysis of methylation pattern and DNAse sensitivity of the plant chromatin containing sequences homologous to the T–DNA may provide answers to some of the pertinent questions.

Many plant species do not contain these homologous sequences. Moreover, the amount of homologous DNA in various species differs greatly (Burner *et al*., 1986). These facts support the hypothesis that the genes may simply represent a genetic scar and may be eventually eliminated from the plant genome. However, the fact that only a single frame shift has occurred in 1957 base-pairs of open reading frame compared to 25 frameshifts in 1240 base-pairs of intergenic region in *N. glauca* compared to present day *A. rhizogenes* indicates that selection may have maintained open reading frames in both the organisms.

Plants regenerated from tissues transformed with *A. rhizogenes* often show an abnormal phenotype. This phenotype is associated with the presence and expression of T_L– DNA in the plants (Tepfer, 1984; Taylor *et al*., 1985b). Such plants frequently revert to a normal phenotype (figure 6). Transcriptional inactivation of the T–DNA is responsible for the phenomenon of reversion (Durand-Tardif *et al*., 1985; Sinkar V. K., unpublished results). The revertant shoots from abnormal plants show very high growth rate and out compete the abnormal shoots. In nature while competing for light and nutrients the revertant shoot would have an advantage. Moreover, the revertant shoots are able to set higher number of viable seeds, and the F_1 progeny plants have normal appearance. These observations can at least partly explain the occurrence of the T–DNA homologous sequences in normal plants.

Acquisition of bacterial genes by higher plants may possibly represent as signify-cant mode of evolution. Analysis of plants for homologous sequences using various other T–DNA probes will help to clear the picture. So far, only a few T–DNAs have been carefully analyzed and fewer have been used for homology study. A few instances of homology between the Ti-plasmid and *N. tabacum* DNA have been reported (Thomashow *et al*., 1980; Yang and Simpson, 1981). These observations have not been pursued further.

Conclusions

While Ri- and Ti–plasmids are clearly related structurally and functionally, and they appear to have evolved from a common ancestor, they have diverged significantly and should be considered separate. Large segments of pRiA4b show little or no homology to the Ti–plasmid. Particularly notable is the absence of homology of the pRiA4b T_L–DNA to any known Ti–plasmids (Hoffman *et al*., 1984).

The Ri T–DNA region can be used for the construction of plant transformation vectors similar to the Ti-plasmid derived vectors (An *et al*., 1985; Klee *et al*., 1985; Zambryski *et al*., 1983). Since the T_L–DNA confers growth abnormalities on plants; deletion of morphogenic loci from the T_L–DNA is essential for general use of the aforementioned vectors. Successful use of unmodified Ri–plasmid to transfer foreign genes to plants has been reported recently (Comai *et al*., 1985; Jensen *et al*., 1986).

A few agronomically important applications of *A. rhizogenes* have been reported to date. Strobel *et al*. (1985) reported increase in the nodulation frequency of alfalfa

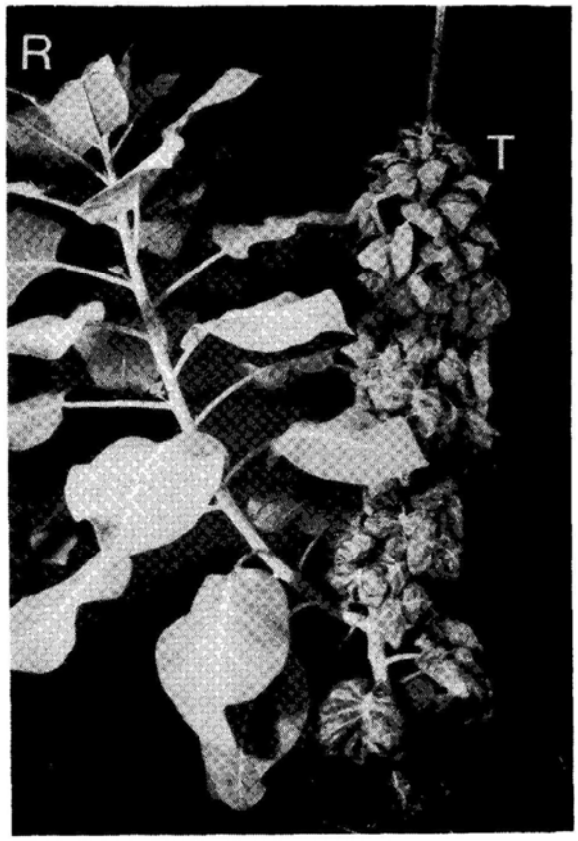


Figure 6. Reversion of transformed *N. tabacum*. *N. glauca* plant regenerated from leaf tissue transformed with *A. rhizogenes*. Note normal appearing revertant shoots (R) emerging from the abnormal (T).

by *Rhizobium meliloti* strains which contain Ri-plasmid. Another interesting application of the bacterium was found in the development of drought-resistance (Strobel and Nachmias, 1985). Application of *A. rhizogenes* to bare root stock of almond trees resulted in a striking increase in root mass after 90 days. Moreover, increases in number of roots, leaves, stem diameter and shoot elongation were seen during the first growing season after the treatment. No pathological reaction was seen with any of the plants used in the study. This approach, therefore, may prove to be useful while transferring drought-sensitive plants from the nursery to the field.

Perhaps the most important contribution of the Ri-plasmid is that it provided impetus to the study of genetic exchange between plants and bacteria and initiated studies on the effects of the exchange of genetic information on evolution of both the groups. At the moment, the Ri-plasmid-plant exchange seems to be the only known system where such an exchange has occurred between the two kingdoms. Genetic exchange between other eukaryotes and bacteria has, however, been postulated in two other instances. It appears that a symbiotic bacterium *Photobacterium leiognathi* has acquired CuZn superoxide dismutase from the host, ponyfish (Martin and

Fridovich, 1981). Similarly, microorganisms isolated from human urine have been reported to produce a material immunologically related to human chorionic gonadotropin (Cohen and Strampp, 1976). *A. rhizogenes* differs from these bacteria at least in one respect, *i.e.*, it does not share any special relationship with the genus *Nicotiana*. In fact, the natural strains of *A. rhizogenes* were isolated from apple or rose (Hedgecock, 1905; Riker *et al.*, 1930; Munneche *et al.*, 1963). Hence, the occurrence of T–DNA homologous sequences in *Nicotiana* species presents a unique case. It is possible that such sequences are present in other plants but have diverged to an extent that they are impossible to detect either with pRiA4b or *Nicotiana glauca* probes.

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