rolA locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants

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Plants containing the left T-DNA (T_L) of *Agrobacterium rhizogenes* show a variety of developmental abnormalities that include severely wrinkled leaves, loss of apical dominance, reduced geotropism of roots, reduced internode distances, and floral hyperstyly. The T_L-DNA also affects the morphology of tumor tissue at the site of inoculation on *Kalanchoe diademontiana* leaves. Single mutations at four loci of the T_L-DNA (*rolA*, *rolB*, *rolC*, and *rolD*) are known to affect tumor morphology on *K. diademontiana* leaves. We regenerated plants from tissues transformed with T_L-DNA containing mutations in each of the *rol* loci in order to determine which of the *rol* loci, if any, control the abnormal plant phenotype. Only plants regenerated after infection with bacteria containing a mutation in *rolA* locus showed loss of the wrinkled leaf phenotype. The *rolA* locus was cloned into the plant transformation vector pGA472 and introduced alone into plants. Transgenic plants containing *rolA* displayed the abnormal phenotype. These results indicate that *rolA* is the primary determinant of the severely wrinkled phenotype of Ri plasmid transgenic plants. Other *rol* loci may influence the degree of developmental abnormalities.

[Key Words: *Agrobacterium rhizogenes*; Ri plasmid; T_L-DNA, *rolA* locus; developmental abnormalities]

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Agrobacterium rhizogenes incites tumor formation on many plants (De Cleene and De Ley 1981; Trulson et al. 1986; Pythoud et al. 1987; Spano et al. 1987). These tumors are characterized on a large number of plant species by initial callus formation and subsequent extensive root proliferation. The induction of callus and root proliferation by *A. rhizogenes* is dependent on the root-inducing (Ri) plasmid (Moore et al. 1979; White and Nester 1980), a portion of which is transferred and stably integrated into the host plant genome (Chilton et al. 1982; White et al. 1982; Willmitzer et al. 1982; for recent reviews, see Sinkar et al. 1987; White and Sinkar 1987). The Ri plasmid present in the agropine-type strain A4 contains two transferred DNA (T-DNA) regions, and although these regions have not been characterized to the extent of the closely related tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*, the Ri T-DNA appears to share many structural features with the Ti T-DNA (Huffman et al. 1984; White et al. 1985). T-DNA transfer from *A. rhizogenes* to plant cells is probably directed by border sequences flanking the T-DNAs similar to Ti T-DNA borders (Slightom et al. 1986), and hybridization and complementation experiments indicate that Ri plasmids contain most of the nontransferred virulence (*vir*) genes (Huffman et al. 1984). Genes present on the two T-DNA regions are transcribed as polyadenylated mRNA when incorpo-

rated into plant nuclear DNA (Durand-Tardif et al. 1985; Taylor et al. 1985a; Ooms et al. 1986b; Sinkar et al. 1988).

The right T-DNA region (T_R-DNA) of the Ri plasmid contains the genes involved in the biosynthesis of indole acetic acid (IAA), a plant hormone, and are referred to as *tms1* and *tms2* (also referred to as *aux1* and *aux2*, respectively). These genes show extensive homology with the *tms* region of the Ti plasmid and can be complemented interspecifically (Huffman et al. 1984; Jouanin 1984; White et al. 1985; Offringa et al. 1986). The T_R-DNA also contains the genes for the synthesis of agropine (Huffman et al. 1984; Jouanin 1984; De Paolis et al. 1984). The nucleotide sequence has been determined for the entire T_L-DNA. A total of 18 open reading frames (ORFs), of 255 nucleotides or greater in length, have been identified (Slightom et al. 1986).

Both T-DNA regions participate in root induction, either individually or together, depending on the plant species or tissue (White et al. 1985; Vilaine and Casse-Delbart 1987). Mutations in the *tms1* and *tms2* genes severely attenuate the root induction response on tobacco stem (*Nicotiana tabacum*), basal carrot disks (*Daucus carota*), and *K. diademontiana* leaves (Cardarelli et al. 1985; White et al. 1985; Boulanger et al. 1986; Vilaine and Casse-Delbart 1987). Four left T-DNA (T_L-DNA) loci have been identified by transposon and deletion mutagenesis, which affects tumor morphology on *K. diademontiana* leaves, and these loci have been ten-

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tatively assigned the names *rolA*, *rolB*, *rolC*, and *rolD* (White et al. 1985; Estramareix et al. 1986). The concerted action of both T_L -DNA and T_R -DNA in some circumstances is shown by the loss of root induction on *K. diagramontiana* leaves when either *tms1* or *rolB* are inactivated (White et al. 1985). Bacteria that contain only the T_R -DNA are capable of inducing roots on *K. diagramontiana* and tobacco stems (White et al. 1985). Recently, bacteria containing T_L -DNA alone have been shown to be capable of inducing root formation on some plant tissue explants and on apical sides of carrot disks (Vilaine and Casse-Delbart 1987; Vilaine et al. 1987). Thus, the Ri plasmid appears to contain multiple mechanisms that can function together or independently to induce root formation on plants.

Plants containing T-DNA can be regenerated from transformed tissues (Ackermann 1977; Spano and Costantino 1982; Costantino et al. 1984; David et al. 1984; Tepfer 1984; Ooms et al. 1985; Taylor et al. 1985b; Sinkar et al. 1987). Such transgenic plants often have developmental abnormalities that are consistently observed in a variety of plant species (Ackermann 1977; Tepfer 1984; Ooms et al. 1985; Taylor et al. 1985a; Sinkar et al. 1987). The transformed phenotype has been correlated with the presence of T_L -DNA (Durand-Tardif et al. 1985; Taylor et al. 1985a; Sinkar et al. 1987), or more precisely, the *EcoRI* fragment 15 (Cardarelli et al. 1987; Spena et al. 1987). Therefore, genes of the T_L -DNA also control the transformed phenotype, as well as morphological effects at primary inoculation sites. In an effort to determine whether the *rol* loci are also involved in the traits of the T_L -DNA containing plants, whole plants were regenerated from tissues transformed with *A. rhizogenes* containing mutations in the T_L -DNA. This analysis indicated that the *rolA* locus is the principle locus controlling the severely wrinkled phenotype of transgenic plants. Furthermore, we introduced the *rolA* locus in plants and found that it alone is sufficient to cause developmental abnormalities in transgenic plants.

Results

Experimental approach

To assess involvement of the *rol* loci on transgenic plant morphology, a set of T_L -DNA transposon and deletion mutations covering the four *rol* loci were selected for plant transformation and regeneration experiments. However, before a set of transgenic plants was derived, a plant-selectable marker in the form of the chimeric kanamycin-resistance gene [eukaryotic kanamycin-resistance (EKR)] was introduced into the T_L -DNA. Plants containing T_L -DNA were obtained by regenerating shoots from roots that appear after infection with bacteria. Although many of the roots developing after an infection by *A. rhizogenes* contain T_L -DNA, not every root necessarily contains it. Frequently, roots not containing T_L -DNA are induced by the auxin-synthesizing genes present in the T_R -DNA. In addition, the prospect that one or more of the mutations might lead to the loss

of the T_L -DNA-mediated root induction or might induce slow-growing abnormal roots was anticipated. Therefore, the EKR gene allowed selection of both abnormal and normal-appearing roots containing the T_L -DNA.

The EKR gene (containing the nopaline synthase promoter, the bacterial neomycin phosphotransferase structural gene, and nopaline synthase 3' sequences) was derived from pMON128 as an *EcoRI* fragment (Fraley et al. 1983) and was cloned into the unique *EcoRI* site of pET33 (Stachel et al. 1985), a defective Tn3 bacterial transposon, giving TnMY903. The defective Tn3 also harbors the β -lactamase gene for resistance to carbenicillin and can transpose when transposase activity is supplied in *trans*. TnMY903 was introduced into a site on the Ri T_L -DNA where previous studies had indicated that an insertion would not disrupt T_L -DNA function, as determined by *K. diagramontiana* leaf inoculation tests. TnMY903 was first introduced into pFW302, a wide host-range cosmid containing the T_L -DNA. An insertion into the left portion of *HindIII* fragment 21 was selected and introduced by homologous recombination into the wild-type Ri plasmid and a set of *A. rhizogenes* strains harboring mutations in *rolA*, *rolB*, *rolC*, and *rolD* (Fig. 1). The resulting strains, R1500 (wild type), R1501 (*rolA*⁻), R1502 (*rolB*⁻), R1503 (*rolC*⁻), and R1504 (*rolD*⁻), were tested for the original mutant phenotype on *K. diagramontiana* leaf inoculations and were observed to produce symptoms characteristic of the *rol* mutation that they represent.

Effects of *rol* mutations on transgenic plants

R1500, R1501, R1502, R1503, and R1504 (Table 1) were used for in vitro leaf disk inoculations to derive a corresponding set of transformed root lines from *N. tabacum* var. *xanthi*. Roots were observed along the edges of the disks within 20 days. The roots were excised and placed on Murashige and Skoog medium without phytohormones containing kanamycin (Murashige and Skoog 1962) (MS⁻). Roots that grew well in the presence of 250 μ g/ml of kanamycin were selected for further study. Untransformed roots could not grow on media containing >50 μ g/ml of kanamycin. Transformed roots were cultured on MS⁻ medium, and shoots that formed spontaneously upon subculture were transferred to fresh plates containing MS⁻ medium and kanamycin. Plants that displayed well-developed shoot and root systems were transplanted into pots.

Plants obtained with R1500 displayed the typical features of T_L -DNA-containing plants. The leaves were highly wrinkled and small; plant height and internode distances were shortened; stems had increased spontaneous adventitious rooting; and plants had a bushier growth habit due to the loss of apical dominance when compared to normal tobacco plants cycled through tissue cultures (Fig. 2). These plants also displayed increased adventitious rooting, a decrease in root geotropic response, and floral hyperstyly (not shown). Plants obtained from infections with strains R1502 (*rolB*⁻), R1503 (*rolC*⁻), and R1504 (*rolD*⁻) all exhibited the abnormal

Figure 1. Restriction map of the T-DNA of pRiA4b. Positions of the *rol* loci, as identified by transposon mutations, are shown (White et al. 1985). Also shown are the positions of the *tms1* and *tms2* loci (Huffman et al. 1984) and the extent of endogenous T-DNA present in *N. glauca* (Furner et al. 1986). Tn5 insertions are indicated by vertical lines. (▼) Insertion of pNos-NPTII-poly(A) Nos (EKR gene) in the left side of H-21; (□) deletion. Restriction fragment used to clone the *rolA* (pFp3) locus is also shown. (For more details on the insertion and deletion mutants used in the study, refer to Table 1.)

phenotype. However, plants containing the R1501 T_L-DNA (*rolA*⁻) did not display the aberrant phenotype of T_L-DNA-containing plants. Particularly evident was the loss of the severely wrinkled leaf trait (see Fig. 3).

Although the plants containing T_L-DNA mutations (with the exception of those with *rolA* mutation) showed overall morphology similar to that displayed by plants containing wild type T_L-DNA, some subtle morphological differences were observed. Similarly, plants

containing T_L-DNA with mutation in *rolA* were not identical in morphology to untransformed plants regenerated from tissue cultures. Therefore, an attempt was made to quantitate some of the different growth behaviors of these plants to assess the contribution of various *rol* genes to the aberrant features. Measurements of height, leaf length-to-width ratio, flower size, and frequency of hyperstily were taken from the different plants (Table 2). Although these data do not delineate

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant phenotype and genotype	Source
<i>Agrobacterium</i> spp.		
R1000 (pRiA4b)	Onc ⁺	derivative of strain A4T (White and Nester 1980)
R1058 (<i>rolA</i> ⁻)	Onc ⁺ , Kan ^R	White et al. (1985)
R1059 (<i>rolB</i> ⁻)	Onc ⁺ , Kan ^R	White et al. (1985)
R1023 (<i>rolB</i> ⁻)	Onc ⁺ , Kan ^R	White et al. (1985)
R1016 (<i>rolC</i> ⁻)	Onc ⁺ , Kan ^R	White et al. (1985)
R1226 (118, <i>rolD</i> ⁻)	Onc ⁺ , Kan ^R	White et al. (1985)
R1500 (R1000 with EKR)	Onc ⁺ , Carb ^R	this study
R1501 (R1058 with EKR)	Onc ⁺ , Kan ^R , Carb ^R	this study
R1502 (R1023 with EKR)	Onc ⁺ , Kan ^R , Carb ^R	this study
R1503 (R1016 with EKR)	Onc ⁺ , Kan ^R , Carb ^R	this study
R1504 (R1226 with EKR)	Onc ⁺ , Kan ^R , Carb ^R	this study
<i>E. coli</i>		
HB101	<i>thr</i> , <i>leu</i> , <i>thi</i> , <i>pro</i>	
C2110	<i>pol</i> , <i>gyrA</i> , <i>Nal</i> ^R	Ditta et al. (1981)
2174 (pPH1j1)	<i>met</i> , <i>pro</i> , <i>Gn</i> ^R , <i>RK2</i> ^{rep} , <i>IncP1</i>	Beringer et al. (1978)
HB101rif (pRK2073)	<i>Tra</i> ⁺	Leong et al. (1982)
Plasmids		
pFW302	<i>Tc</i> ^R , <i>RK2</i> ^{rep} , <i>IncP1</i>	H-11 to H-18 of pRiA4b in pVK102 (White et al. 1985)
pFW41	<i>Tc</i> ^R , <i>RK2</i> ^{rep} , <i>IncP1</i>	H-24a to H-3 in pHK17 (Huffman et al. 1984)
pMY903	Carb ^R	pNOS-NPTII-3'NOS (EKR) into pETW3, (M. Yanofsky, unpubl.)
pVS1	<i>Tet</i> ^R , Carb ^R	EKR into H-21 (this study)
pGA472	<i>Tet</i> ^R	Ti plasmid-derived vector for plant transformation (An et al. 1985)
pFP3	<i>Tet</i> ^R	<i>rolA</i> into pGA 472

Figure 2. *N. tabacum* regenerates from tissue culture. (Plant A) Regenerated from R1500-transformed leaf tissue of *N. tabacum*. (Plant B) *N. tabacum* regenerated from nontransformed tissues.

clear differences between *rol* mutants, the results for plants with *rolB*⁻ and *rolD*⁻ T-DNA suggest that these two genes may exert some control over the severity of abnormal phenotype in the presence of *rolA*. Plants with *rolC*⁻ T_L-DNA were most similar in appearance to plants with wild-type T-DNA. On the other hand, *rolA*⁻ plants were not identical to the control plants regenerated from untransformed tissues. The *rolA*⁻ plants were consistently different in height, leaf length/width ratio, flower size, and frequency of hyperstyly when compared to normal plants. The reasons for these differences are unknown, but other T_L-DNA genes, possibly *rolB*, *rolC*, and *rolD*, or T_R-DNA may be responsible for these differences.

The presence of T-DNA sequences in the transgenic plants was confirmed by DNA hybridization analysis of genomic DNA separately for T_L- and T_R-DNA. The genomic DNA was analyzed with both *Hind*III and *Eco*RI, although only a representative sample of the results is shown (Fig. 4). In all cases, kanamycin-resistant plants appeared to contain at least one copy of the T_L-DNA, as indicated by the detection of fragments that comigrated with the fragments from the reconstruction experiment. Internal T_L-DNA fragments were observed for *Hind*III fragment 17 in wild type and all mutants except *rolC*⁻ plants, where the *rolC* mutation disrupts the fragment. Internal frequent *Hind*III-32 was present in all lines, except one containing *rolD*⁻ where the fragment was deleted in the Ri plasmid. Likewise, *rolB*⁻ plants appeared to be missing only *Hind*III-30a, which is disrupted by the Tn5 insertion. The chimeric kanamycin-resistance gene and the *rolA* insertions disrupted the *Hind*III-21 fragment of the wild-type T_L-DNA and created new fragments of high molecular weight (11 kb). The high molecular weight of this band and the fact that this fragment comigrates with the related endogenous T-DNA sequences makes analysis of the *rolA* locus with *Hind*III difficult. However, the *rolA* locus was analyzed in some

lines with *Eco*RI, and these data corroborated the evidence with *Hind*III that each line contains at least one copy of the T_L-DNA. These data demonstrated that the lack of plant abnormalities in the case of *rolA*⁻ lines are not due to the absence of T_L-DNA transfer.

The hybridization data also show that all lines contain at least some portion of the T_R-DNA. The results indicate that some of the lines contain the auxin genes, as indicated by the presence of the fragments in two lines that comigrate with *Hind*III-15. One of the *rolC*⁻ lines (lane 5, Fig. 3B) contains an exceptionally long T_R-DNA that extends beyond the normal boundary in the left end of *Hind*III-22. The effect of the auxin genes on the plant morphology is not clear; however, the presence of the severely wrinkled leaf phenotype is not correlated with the presence of the complete T_R-DNA. For example, the DNA from an R1500 transformed plant did not contain the complete *tms1* region of the T_R-DNA (lane 2, Fig. 4). This observation is consistent with earlier observations (Durand-Tardif et al. 1985; Taylor et al. 1985b).

Characterization of *rolA* locus

Studies performed with T_L-DNA mutants indicated that the *rolA* locus probably controls the severely aberrant plant phenotype. However, whether *rolA* locus alone controls this plant phenotype or acts in concert with other unidentified genes was not clear. If the phenotype was controlled by the single locus, the introduction of this locus into plants without other T_L-DNA genes should result in aberrant plants. Genetic studies have shown that the *rolA* locus spans across the central *Hind*III site, between *Hind*III-21 and *Hind*III-30a fragments, and is defined by the *Eco*RI site on the left and the *Bam*HI site on the right (Fig. 1; White et al. 1985).

The *rolA* locus was subcloned as the 2-kb *Eco*RI/*Bam*HI fragment into the plant transformation vector pGA472 (An et al. 1985) and named pFP3. The vector pGA472 was derived from the nopaline-type Ti plasmid pTiT37 and does not contain any pRiA4b T_L-DNA. The vector is capable of autonomous replication in *Agrobac-*

Figure 3. Comparison of leaves of the plants transformed with *rol* loci mutants. From left: untransformed, R1500 (wild type), R1501 (*rolA*⁻), R1502 (*rolB*⁻), R1503 (*rolC*⁻), R1504 (*rolD*⁻).

Table 2. Comparison of transgenic plants

	Control	R1500	<i>rolA</i> ⁻	<i>rolB</i> ⁻	<i>rolC</i> ⁻	<i>rolD</i> ⁻
Height (cm)	120 (10)	55 (19)	92 (12)	83 (14)	63 (5)	77 (6)
Leaf length ^a /width ^b	1.80 (0.08)	1.10 (0.20)	1.50 (0.15)	1.40 (0.13)	1.20 (0.07)	1.50 (0.13)
Flower size ^c (cm)	4.10 (0.06)	3.00 (0.29)	3.60 (0.216)	3.20 (0.06)	3.30 (0.12)	3.30 (0.09)
Hyperstyly (%)	0	70	17 ^d	53	38 ^d	45

Figures in parentheses indicate standard deviation.

^a Maximum distance between two points parallel to the mid-vein of the leaf; 15 leaves per determination.

^b Maximum distance between two points perpendicular to the mid-vein of the leaf.

^c Length of the corolla.

^d Some flowers showed shorter style (>1 mm) than the anthers (not considered for hyperstyly measurements).

terium (binary vector) and contains the plant-expressed kanamycin-resistance gene. The plasmid pFP3 was mobilized into the T-DNA-deficient strain of *A. tumefaciens* LBA4404 (Hoekema et al. 1983). Leaf disks of *N. tabacum* were transformed with LBA4404 containing pFP3, and plants were selected on MS104 medium containing 100 µg/ml kanamycin (Horsch et al. 1985).

Plants transformed with pFP3 had the aberrant phenotype similar to T₁-DNA containing plants (data not

shown). The plants were severely stunted as an apparent result of extremely shortened internode distances. The leaf morphology was also extremely altered. The length-to-width ratio was very low (0.95) compared to that of normal plant leaves, and the leaves were severely wrinkled in appearance.

A T-DNA analysis was performed with total genomic DNA extracted from *rolA* transgenic plants. A 3.2-kb internal T-DNA fragment extending from one *Hind*III site

Figure 4. T-DNA analysis of the transgenic plants. DNA isolated from transgenic *N. tabacum* plants was digested with *Hind*III, separated electrophoretically on gel, transferred to nitrocellulose membranes, and probed with pFW302 (A) and pFW41 (B). (Lanes 1) One-copy reconstruction using the Ri plasmid and DNA of untransformed *N. tabacum*; (lanes 2) transformed with R1500; (lanes 3) transformed with R1504; (lanes 4) transformed with R1503; (lanes 5) transformed with R1502; (lanes 6) transformed with R1501.

within the *rolA* locus and a second site within the pGA472 vector was used as evidence for the presence of the *rolA* region. The plant DNA was digested with *Hind*III and probed with Ri T_L-DNA sequences, and the 3.2-kb fragment was observed in all plants with the aberrant phenotype (Fig. 5).

Because the *Eco*RI–*Bam*HI fragment alone can cause developmental abnormalities in transgenic plants, the fragment was sequenced to obtain information about gene(s) and their protein product(s). Previous studies have indicated that the carboxyl region of the *rolB* locus extends to the left of the *Pst*I site present in *Hind*III-30a, going toward the central *Hind*III site between *Hind*III-21 and *Hind*III-30a (Furner et al. 1986). Hence, the locus (loci) responsible for the aberrant morphology should be situated on the *Eco*RI–*Pst*I fragment. The DNA sequence of this region showed the longest ORF extending into *Hind*III-30a from *Hind*III-21. The ORF is 300 nucleotides in length and potentially encodes for a protein of 11,015 daltons (Fig. 6). No significant homology was

detected between this *rolA* protein and other T-DNA or any other gene products. We also mapped the Tn5 insertion in R1058, the *rolA*[−] strain, and found that the insertion was indeed within the coding region of the *rolA* locus (Fig. 6).

Discussion

The T_L-DNA of the Ri plasmid from *A. rhizogenes* strain A4 has been shown to control several phenotypic behaviors in transgenic plants and primary wound tumors. These results clearly demonstrate the involvement of at least one T_L-DNA locus, namely *rolA*, in the expression of phenotypic traits in both transgenic plants and primary tumors. *rolA* had been shown previously to affect the morphology of roots that proliferate from *K. diagemontiana* leaf tumors. *K. diagemontiana* roots induced by strains containing *rolA* tend to be thicker, more curled, and more stunted than roots induced by a *rolA*[−] strain (White et al. 1985). A recent report indicates that *rolA* may also be able to direct root initiation from tobacco stem explants and leaf disks (Vilaine et al. 1987). *rolA* alone controls the severely wrinkled leaf morphology of transgenic plants, as demonstrated by the leaf morphology of plants containing the *rolA* locus without other T_L-DNA genes. In addition, no severely wrinkled plants were obtained with a T_L-DNA containing a *rolA* mutation; therefore, no other T_L-DNA gene appears to be capable of directing the phenotype.

The wrinkled leaf effect is probably not limited to tobacco. Similar results have been obtained with *Nicotiana glauca* containing the *rolA* gene (P. Pythoud and V.S. Sinkar, unpubl.), and the wrinkled leaf phenotype has been reported in a range of plants including carrot (*D. carota*), morning glory (*Convolvulus arvensis*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), and *Nicotiana plumbaginifolia* (Tepfer 1984; Ooms et al. 1985; Peerbolte 1986; Shahin et al. 1986).

The analysis of plants containing individual T_L-DNA genes is affording a better understanding of the contribution to the seemingly complex and pleiotropic effects of the Ri T_L-DNA. The severely wrinkled leaf morphology that is controlled by *rolA* corresponds to the T' phenotype of Tepfer (1984). Individual regenerated plants often display the wrinkled (T') phenotype or less severely affected symptoms (T phenotypic plants; Tepfer 1984). The T'/T switching has been observed previously with plants containing the complete T_L-DNA (Tepfer 1984) or plants with *rol* loci A, B, and C (Spena et al. 1987). These results and the mRNA analysis of revertant plants (Sinkar et al. 1988) indicate that the switching of phenotypes can result from the loss or reduction in the expression of *rolA*. Loss of the wrinkled plant phenotype correlates with the absence of a *rolA*-specific transcript (Sinkar et al. 1988). The size of the *rolA* transcript has been estimated at 650–800 kb (Taylor et al. 1985a; Spena et al. 1987; Sinkar et al. 1988). The fact that plants containing *rolA* alone can revert to normal suggests that reversion or switching is due to a host plant function that leads to inactivation of the gene. Whether

Figure 5. T-DNA analysis of the plants transformed with *rolA*. DNA isolated from a plant transformed with *rolA* was digested with *Hind*III and transferred to nitrocellulose membrane after electrophoretic separation on agarose. The probe used was pFW302. (Top) The restriction map shows the T-DNA of pFP3. (B_L and B_R). The left and the right borders respectively, of the T-DNA of the Ti plasmid. The *rolA* locus [isolated as the *Eco*RI–*Bam*HI fragment from the Ri plasmid (Fig. 1)] is shown as an open box. The DNA fragment of 3.2-kb expected to hybridize with pFW302 is indicated by an arrow. (Lane 1) One-copy reconstruction; (lane 2) untransformed *N. tabacum*; (lane 3) transformed *N. tabacum* with *rolA*.

ATCATTGCGTAGACTGCATATTGACCGCTCTTCGGCGCTGGGCTGCTTTTACTGTTGC
 CTAGCGTTCGGACAGCCAGAGAAATGGGCTATATAGATCCTTTCATCAAAACAAAACA
 TTACTAAGATCATGCTGTAAACGCTTCAATACGGTGAGTGTGGTTGTAGGTTCAATTATTA
 CTATTTTGAAGCTGTGATTTCCCTTTTCTAATAATGCACCTATTTTCATGTTTCAGAAT
 Met
 GGAATTAGCCGGACTAAACGTCGCGCGGATGGCCAGACCTTCGGAGTATTATCGCTCGT
 GluLeuAlaGlyLeuAsnValAlaGlyMetAlaGlnThrPheGlyValLeuSerLeuVal
 CTGTTCTAAGCTTGTAGGCTGCAAAGGCCAAGAGGAAGGCCAAACGGGTATCCCGGG
 CysSerLysLeuValArgArgAlaLysAlaLysArgLysAlaLysArgValSerProGly
 CGAACGCGACCATCTTGCTGAGCCAGCCAATCTGAGCACCCTCTTGGCCATGACTTC
 GluArgAspHisLeuAlaGluProAlaAsnLeuSerThrThrProLeuAlaMetThrSer
 CCAAGCCGACCGGGACGTTCAACGACCCGCGAGTTGCTGCGAAGGGACCTTTGTGCGC
 GlnAlaArgProGlyArgSerThrThrArgGluLeuLeuArgArgAspProLeuSerPro
 GGACGTGAAATTCAGACCTACGGGATTAAATACGATTTCGAAACAAACCTACGGGATTA
 AspValLysIleGlnThrTyrGlyIleAsnThrHisPheGluThrAsnLeuArgAsp***
 ATACGCACGTGGCTGGCGGTCTTCGATTCTTCCACGCCGAGATGATATCGAATATGT
 TCTGTTAAGTTAAATAAGCTGCGAGCCATGGCGGATTGTCTCTTTTATTAATATAGT
 ACTTAAACGTCTCTTTAGACGCTTTGTGTAATGTGCGTAAATGTTTATGTCAAATGTA

Figure 6. Nucleotide sequence of *rolA* locus. The nucleotide sequence of the relevant part of the *EcoRI*–*PstI* fragment is presented (720 bp). Three-letter amino acid code is placed below the codons in the ORF. Putative promoter elements and polyadenylation signal sites are underlined. The site of Tn5 insertion in the *rolA* mutant used in these studies is indicated by an arrow.

switching is always due to changes in *rolA* expression is unknown.

The presence of the severely wrinkled and less severe phenotypes when plants contain the complete T_L -DNA, or *rolA*, *rolB*, and *rolC* loci, indicates that other *rol* genes, particularly *rolB* and *rolC*, also contribute to aspects of the abnormal plant phenotypes. Effects in transgenic plants by other *rol* loci are also suggested by subtle differences of *rol* mutant plants. *rolA*[−] plants were not identical to normal plants in appearance, nor were *rolB*[−] and *rolD*[−] identical to complete T_L -DNA plants. However, the nature of the *rol* mutation plants is such that we cannot confidently assign phenotypes to the other genes. Individual plants varied, depending on the copy number of the T-DNA insert and in the presence of T_R -DNA sequences. A better assessment of the other genes will come from analyses similar to those using the *rolA* plants. Studies with transgenic plants containing the other *rol* genes, either individually or in combination, are in progress, and at least one recent report concerning the effect of another *rol* locus (*rolB*) in transgenic plants has appeared. Cardarelli et al. (1987) reported that plants containing the *rolB* locus have the wrinkled phenotype, although no distinction is made between the T and T' phenotype (slightly wrinkled vs. severely wrinkled). In similar experiments, we have observed some minor abnormalities such as leaves with uneven surfaces, increased leaf hairs, reduction in plant height, and floral hyperstyly in plants containing *rolB*, *rolC*, or *rolD* (P. Phythoud, unpubl.). In fact, abnormalities of various degrees were observed in plants containing only vector DNA (pGA472). Culture conditions also appear to affect the less conspicuous plant abnor-

malities. A careful evaluation of transgenic plants harboring individual T_L -DNA genes will shed further light on the contributions of each locus.

Aside from the phenotype of transgenic plants, several recent reports have demonstrated that different *rol* loci may direct similar plant growth responses. Vilaine et al. (1987) have reported that leaf disks inoculated with strains containing *rolA* alone produce roots, and these roots behave in an identical fashion to roots harboring the complete T_L -DNA. Spena et al. (1987) report that either *rolA* or *rolB* will induce rooting on tobacco leaf disks, and *rolC* will induce rooting if provided with the cauliflower mosaic virus ³⁵S promoter. (These reports did not include descriptions of whole plant regeneration and morphology for the individual genes.) On the other hand, only *rolB* in combination with the auxin biosynthesis genes was able to induce roots on *K. diargremon-tiana* leaves (Spena et al. 1987; F. Shaheen and F.F. White, unpubl.). Thus, each *rol* loci and possibly other genes of the T_L -DNA (e.g., ORF 13 effects on carrot disks; Cardarelli et al. 1987) appear to perform some biochemical functions which, in certain circumstances, result in alterations of plant growth and differentiation. The visible effects of these alterations appear to vary, depending on the host plant or tissues. The proteins and the associated biochemical activities remain unknown.

Specifically, with regard to the *rolA* locus, the nucleotide sequence of the region has revealed that the largest ORF corresponds to a polypeptide of 100 amino acid residues and 11,015 daltons. This finding is in complete agreement with the ORF (ORF 10) identified by Slightom et al. (1986). (Slightom et al. reported a potential peptide, 99 amino acids long, although the sequence presented indicated the same 100-amino-acid protein as in our results.) The sequence of one Tn5 mutant places the insertion within ORF 10. In addition, as reported previously, an insertion at the *HindIII* site within ORF 10 resulted in a *rolA* phenotype. However, smaller ORFs exist within the region of the T_L -DNA used in these experiments and in the experiments of others, and the possibility that an alternative ORF encodes the *rolA* function has not been excluded. We have made gene constructions that will demonstrate conclusively which ORFs complement *rolA* mutations on *K. diargremon-tiana* leaf inoculations and yield the severely wrinkled leaf morphology in regenerated plants. These experiments are in progress.

The intriguing question remains as to the mode of action of the *rolA* and other *rol* loci. Although little is known regarding biochemical properties of Ri-transformed plants compared to normal plants, the phenotypes suggest that the pleiotropic effects involve disturbances in phytohormone signaling. Ri-transformed potato plants have also been reported to have disturbances in cellular electrolyte concentrations (Ooms et al. 1986a). These observations are likely to stem from a common cause because membrane permeability and electrolyte flux are intimately connected with phytohormone flux. The effects of the *rol* genes and the absence of sequence relatedness to known Ti T-DNA plant oncogenes suggest that the *rol*-gene functions will repre-

sent previously undescribed modes of regulation of plant growth and development.

Material and methods

Bacterial strains and plasmids

A. rhizogenes and *E. coli* strains and plasmids used in the study are listed in Table 1. *Agrobacterium* strains were maintained on AB minimal agar (White et al. 1985), supplied with the appropriate antibiotic when necessary. Antibiotic concentrations used were 100 µg/ml kanamycin (Sigma Chemical Company), 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 tetracycline (Sigma), or 50 µg/ml nalidixic acid (Sigma).

Bacterial DNA isolation

E. coli clones were screened for plasmid by the method of Birnboim and Doly (1979). Large amounts of plasmid DNA were prepared by the scaleup of this procedure and purified by cesium chloride–ethidium bromide gradient centrifugation. The ethidium bromide was removed by extraction with *n*-butanol saturated with water, and the DNA was precipitated with two volumes of 70% ethanol. The *Agrobacterium* DNA was isolated as described previously (White et al. 1985).

Molecular cloning

Construction of the suicidal plasmid TnMY903 by cloning a plant kanamycin-resistance cartridge [pNOS-NPTII-poly(A) NOS] obtained from pMON128 (Fraley et al. 1983) into an *EcoRI* site of a disarmed Tn3 vector has been described in the text. Cloning of *rolA* locus into a disarmed Ti plasmid vector, pGA472 (An et al. 1985) has also been described in the text. The restriction fragment of the T_i-DNA used for the cloning is shown in Figure 1.

Transposon mutagenesis

Tn3 insertions (carbenicillin resistance) were generated by tetraparental mating, using *E. coli* C2110 (*polA*[−]) containing pFW302, *E. coli* HB101 containing TnMY903, *E. coli* HB101 containing pRK2073, and *E. coli* HB101 harboring pSShe (transposons on a plasmid with ColE1 replicon; Stachel et al. 1985). Transconjugants were selected on L agar containing carbenicillin and nalidixic acid. Replications of pRK2073, TnMY903, and pSShe are strictly dependent on the *polA*-gene product. pFW302 containing the Tn3 insertion to the left of the *rolA* locus in *HindIII* 21 (pVS1) was selected for homologous recombination with the wild-type and various Ri plasmid *rol* mutants.

Bacterial conjugation and homologous recombination

The mobilizable cosmid (pVS1) was introduced into *A. rhizogenes* strains by triparental mating procedure, as described by White et al. (1985). The self-conjugal plasmid pPH1JI was introduced into *A. rhizogenes* transconjugant strains to select for homologous recombination, as described previously (White et al. 1985).

DNA sequencing

Small fragments of the *EcoRI*–*PstI* fragment were cloned into M13 and were sequenced by the dideoxy method.

Plant inoculations, tissue propagation, and regeneration

Disks obtained from leaves, between the fourth and sixth position from the apex, were used for the transformation of *N. tabacum* var. *xanthi*. The disks were infected with *A. rhizogenes* strains according to the method of Horsch et al. (1985). Roots formed on the disks were excised and were grown on MS[−] medium containing kanamycin (250 µg/ml). These roots regenerated into plants.

For examining the effect of the *rolA* locus on the phenotype of the regenerated plants, *N. tabacum* leaf disks were infected with *A. tumefaciens* LBA 4404 (Ooms et al. 1985; a helper strain that provides the *vir* functions in *trans*) containing *rolA* (pFP3). The leaf disks were grown on MS 104 growth medium (Horsch et al. 1985) containing kanamycin (100 µg/ml). Kanamycin-resistant shoots formed from these disks were excised and grown on MS[−] medium supplemented with kanamycin.

Plant nucleic acid isolation and T-DNA analysis

Plant DNA isolation and T-DNA analysis was performed, as described previously (Sinkar et al. 1988).

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rolA locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants.

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