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

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Plants containing the left T-DNA (Tl) 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 Tl-DNA also affects the morphology of tumor tissue at the site of inoculation on Kalanchoe diurumontiana leaves. Single mutations at four loci of the Tl-DNA (rolA, rolB, rolC, and rolD) are known to affect tumor morphology on K. diurumontiana leaves. We regenerated K. diurumontiana also affects the morphology of tumor tissue at TL-DNA reduced intemode distances, and floral hyperstyly. The loci show a variety of developmental abnormalities. Agrobacterium rhizogenes Plants containing the left T-DNA (Tl) of Agrobacterium rhizogenes show a variety of developmental abnormalities in transgenic tobacco plants. Other rol loci may influence the degree of developmental abnormalities. [Key Words: Agrobacterium rhizogenes, Ri plasmid, Tl-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 incorporated 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 Tl-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. diurumontiana leaves (Cardarelli et al. 1985; White et al. 1985; Boulanger et al. 1986; Vilaine and Casse-Delbart 1987). Four left T-DNA (Tl-DNA) loci have been identified by transposon and deletion mutagenesis, which affects tumor morphology on K. diurumontiana leaves, and these loci have been ten.
Experimentally assigned the names rolA, rolB, rolC, and rolD [White et al. 1985; Estromareix et al. 1986]. The concerted action of both T₁-DNA and T₉-DNA in some circumstances is shown by the loss of root induction on K. diagremontiana leaves when either tms1 or rolB are inactivated [White et al. 1985]. Bacteria that contain only the T₉-DNA are capable of inducing roots on K. diagremontiana and tobacco stems [White et al. 1985]. Recently, bacteria containing T₁-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. 1985a; 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 T₁-DNA [Durand-Tardif et al. 1985; Taylor et al. 1985a; Sinkar et al. 1987] or, more precisely, the EcoRl fragment 15 [Cardarelli et al. 1987; Spena et al. 1987]. Therefore, genes of the T₁-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 T₁-DNA-containing plants, whole plants were regenerated from tissues transformed with A. rhizogenes containing mutations in the T₁-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 T₁-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 T₁-DNA. Plants containing T₁-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 T₁-DNA, not every root necessarily contains it. Frequently, roots not containing T₁-DNA are induced by the auxin-synthesizing genes present in the T₉-DNA. In addition, the prospect that one or more of the mutations might lead to the loss of the T₁-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 T₁-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 EcoRl fragment [Fraley et al. 1983] and was cloned into the unique EcoRl 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 T₁-DNA where previous studies had indicated that an insertion would not disrupt T₁-DNA function, as determined by K. diagremontiana leaf inoculation tests. TnMY903 was first introduced into pFW302, a wide host-range cosmid containing the T₁-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. diagremontiana 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 μg/ml of kanamycin were selected for further study. Untransformed roots could not grow on media containing >50 μg/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 T₁-DNA-containing plants. The leaves were highly wrinkled and small, plant height and internode distances were shortened, stems had increased 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 -) also exhibited the abnormal

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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 (Fumer 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.)

Table 1. Bacterial strains and plasmids used in this study

| Strains and plasmids | Relevant phenotype and genotype | Source |
|----------------------|--------------------------------|--------|
| **Agrobacterium spp.** |                                 |        |
| R1000 [pRiA4b]       | Onc+                           | derivative of strain A4T (White and Nester 1980) |
| R1058 [rolA−]        | Onc+, KanR                     | White et al. (1985) |
| R1059 [rolB−]        | Onc+, KanR                     | White et al. (1985) |
| R1023 [rolB−]        | Onc+, KanR                     | White et al. (1985) |
| R1016 [rolC−]        | Onc+, KanR                     | White et al. (1985) |
| R1226 [118, rolD−]   | Onc+, KanR                     | White et al. (1985) |
| R1500 [R1000 with EKR] | Onc+, CarbR                  | this study |
| R1501 [R1058 with EKR] | Onc+, KanR, CarbR             | this study |
| R1502 [R1023 with EKR] | Onc+, KanR, CarbR             | this study |
| R1503 [R1016 with EKR] | Onc+, KanR, CarbR             | this study |
| R1504 [R1226 with EKR] | Onc+, KanR, CarbR             | this study |

| E. coli              |                                 |        |
|----------------------|--------------------------------|--------|
| HB101                | thr, leu, thi, pro             | Ditta et al. (1981) |
| C2110                | pol, gyrA, NalR                | Beringer et al. (1978) |
| 2174 [pPH1]j]       | met, pro, Gna, RK2\(^{\text{II}}\), IncP1 | Leong et al. (1982) |
| HB101rif (pRK2073)   | Tra+                           |        |

| **Plasmids**         |                                 |        |
|----------------------|--------------------------------|--------|
| pFW302               | TcR, RK2\(^{\text{II}}\), IncP1 | H-11 to H-18 of pRiA4b in pVK102 (White et al. 1985) |
| pFW41                | TcR, RK2\(^{\text{II}}\), IncP1 | H-24a to H-3 in pHK17 (Huffman et al. 1984) |
| pMY903               | TcR, CarbR                     | pNOS-NPTII-3'NOS (EKR) into pETW3, (M. Yanofsky, unpubl.) |
| pVS1                 | TetR, CarbR                    | EKR into H-21 (this study) |
| pGA472               | TetR                           | Ti plasmid-derived vector for plant transformation (An et al. 1985) |
| pFP3                 | TetR                           | rolA into pGA 472 |

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 hyperstlyy were taken from the different plants (Table 2). Although these data do not delineate

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**Table 2.** Relevant phenotype and genotype sources

| Strains and plasmids | Relevant phenotype and genotype | Source |
|----------------------|--------------------------------|--------|
| **Agrobacterium spp.** |                                 |        |
| R1000 [pRiA4b]       | Onc+                           | derivative of strain A4T (White and Nester 1980) |
| R1058 [rolA−]        | Onc+, KanR                     | White et al. (1985) |
| R1059 [rolB−]        | Onc+, KanR                     | White et al. (1985) |
| R1023 [rolB−]        | Onc+, KanR                     | White et al. (1985) |
| R1016 [rolC−]        | Onc+, KanR                     | White et al. (1985) |
| R1226 [118, rolD−]   | Onc+, KanR                     | White et al. (1985) |
| R1500 [R1000 with EKR] | Onc+, CarbR                  | this study |
| R1501 [R1058 with EKR] | Onc+, KanR, CarbR             | this study |
| R1502 [R1023 with EKR] | Onc+, KanR, CarbR             | this study |
| R1503 [R1016 with EKR] | Onc+, KanR, CarbR             | this study |
| R1504 [R1226 with EKR] | Onc+, KanR, CarbR             | this study |

| **Plasmids**         |                                 |        |
|----------------------|--------------------------------|--------|
| pFW302               | TcR, RK2\(^{\text{II}}\), IncP1 | H-11 to H-18 of pRiA4b in pVK102 (White et al. 1985) |
| pFW41                | TcR, RK2\(^{\text{II}}\), IncP1 | H-24a to H-3 in pHK17 (Huffman et al. 1984) |
| pMY903               | TcR, CarbR                     | pNOS-NPTII-3'NOS (EKR) into pETW3, (M. Yanofsky, unpubl.) |
| pVS1                 | TetR, CarbR                    | EKR into H-21 (this study) |
| pGA472               | TetR                           | Ti plasmid-derived vector for plant transformation (An et al. 1985) |
| pFP3                 | TetR                           | rolA into pGA 472 |
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-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 hyperstlyly when compared to normal plants. The reasons for these differences are unknown, but other T-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 TL- and TR-DNA. The genomic DNA was analyzed with both HindIII and EcoRI, 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-DNA, as indicated by the detection of fragments that comigrated with the fragments from the reconstruction experiment. Internal T-DNA fragments were observed for HindIII fragment 17 in wild type and all mutants except rolC" plants, where the rolC mutation disrupts the fragment. Internal frequent HindIII-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 HindIII-30a, which is disrupted by the Tn5 insertion. The chimeric kanamycin-resistance gene and the rolA insertions disrupted the HindIII-21 fragment of the wild-type T-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 HindIII difficult. However, the rolA locus was analyzed in some lines with EcoRI, and these data corroborated the evidence with HindIII that each line contains at least one copy of the T-DNA. These data demonstrated that the lack of plant abnormalities in the case of rolA" lines are not due to the absence of T-DNA transfer.

The hybridization data also show that all lines contain at least some portion of the T-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 HindIII-15. One of the rolC" lines (lane 5, Fig. 3B) contains an exceptionally long T-DNA that extends beyond the normal boundary in the left end of HindIII-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-DNA. For example, the DNA from an R1500 transformed plant did not contain the complete tms1 region of the T-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-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-DNA genes should result in aberrant plants. Genetic studies have shown that the rolA locus spans across the central HindIII site, between HindIII-21 and HindIII-30a fragments, and is defined by the EcoRI site on the left and the BamHI site on the right (Fig. 1; White et al. 1985).

The rolA locus was subcloned as the 2-kb EcoRI/BamHI 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 pRi44b T-DNA. The vector is capable of autonomous replication in Agrobac-
Table 2. Comparison of transgenic plants

|                  | Control | R1500 | roiA−  | roiB−  | roiC−  | roiD−  |
|------------------|---------|-------|--------|--------|--------|--------|
| Height [cm]      | 120     | 55    | 92     | 83     | 63     | 77     |
| (10)             | (19)    | (12)  | (14)   | (5)    | (6)    |
| Leaf length×/width× | 1.80    | 1.10  | 1.50   | 1.40   | 1.20   | 1.50   |
| (0.08)           | (0.20)  | (0.15)| (0.13) | (0.07) | (0.13) |
| Flower size× (cm) | 4.10    | 3.00  | 3.60   | 3.20   | 3.30   | 3.30   |
| (0.06)           | (0.29)  | (0.216)| (0.06) | (0.12) | (0.09) |
| Hyperstly (%)    | 0       | 70    | 174    | 53     | 384    | 45     |

Figures in parentheses indicate standard deviation.

* Maximum distance between two points parallel to the mid-vein of the leaf, 15 leaves per determination.

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

Length of the corolla.

Some flowers showed shorter style (>1 mm) than the anthers (not considered for hyperstly 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 [Hoeckema 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 T-L-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 HindIII site

Figure 4. T-DNA analysis of the transgenic plants. DNA isolated from transgenic N. tabacum plants was digested with HindIII, 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 HindIII and probed with Ri T\textsubscript{L}-DNA sequences, and the 3.2-kb fragment was observed in all plants with the aberrant phenotype (Fig. 5).

Because the EcoRI–BamHI 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 PstI site present in HindIII-30a, going toward the central HindIII site between HindIII-21 and HindIII-30a [Furner et al. 1986]. Hence, the locus (loci) responsible for the aberrant morphology should be situated on the EcoRI–PstI fragment. The DNA sequence of this region showed the longest ORF situated on the EcoRI–PstI fragment. The DNA sequence in R1058, the rolA· strain, and found that the insertion was indeed within the coding region of the rolA locus (Fig. 6).

**Figure 5.** T-DNA analysis of the plants transformed with rolA. DNA isolated from a plant transformed with rolA was digested with HindIII 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. [\textit{B}_L and \textit{B}_R]. The left and the right borders respectively, of the T-DNA of the Ti plasmid. The rolA locus [isolated as the EcoRI–BamHI 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 \textit{N. tabacum}; (lane 3) transformed \textit{N. tabacum} with rolA.

Discussion

The T\textsubscript{L}-DNA of the Ri plasmid from \textit{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\textsubscript{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 \textit{K. diammontiana} leaf tumors. \textit{K. diammontiana} 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\textsubscript{L}-DNA genes. In addition, no severely wrinkled plants were obtained with a T\textsubscript{L}-DNA containing a rolA mutation; therefore, no other T\textsubscript{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 \textit{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 [\textit{D. carota}], morning glory [\textit{Convolvulus arvensis}], tomato [\textit{Lycopersicum esculentum}], potato [\textit{Solanum tuberosum}], and \textit{Nicotiana plumaginifolia} (Teper 1984, Ooms et al. 1985; Peerbolte 1986; Shahin et al. 1986).

The analysis of plants containing individual T\textsubscript{L}-DNA genes is affording a better understanding of the contribution to the seemingly complex and pleiotropic effects of the Ri T\textsubscript{L}-DNA. The severely wrinkled leaf morphology that is controlled by rolA corresponds to the T' phenotype of Teper [1984]. Individual regenerated plants often display the wrinkled (T') phenotype or less severely affected symptoms (T phenotypic plants; Teper 1984). The T'/T switching has been observed previously with plants containing the complete T\textsubscript{L}-DNA (Teper 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 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 Ti-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 Ti-DNA plants. However, the nature of the rol mutation implies that such mutations 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-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 hyperstevy 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 abnormalities. A careful evaluation of transgenic plants harboring individual Ti-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 Ti-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. diamentiana leaves [Spena et al. 1987; F. Shaheen and F.F. White, unpubl.]. Thus, each rol locus and possibly other genes of the Ti-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 roiA phenotype. However, smaller ORFs exist within the region of the Ti-DNA used in these experiments and in the experiments of others, and the possibility that an alternative ORF encodes the roiA function has not been excluded. We have made gene constructions that will demonstrate conclusively which ORFs complement rolA mutations on K. diamentiana 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 T2-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 [transposes on a plasmid with ColEl 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-conjugating plasmid pPH11 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 dyeoxy 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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