Early flowering in transgenic tobacco plants possessing the *rolC* gene of *Agrobacterium* *rhizogenes* Ri plasmid

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ABSTRACT

Progeny plants obtained by self-crossing transgenic tobacco plants possessing the *rolC* gene (ORF 12 of TL-DNA of Ri plasmid) showed stable inheritance of dwarfness in plant height as well as in floral organs. A histological analysis of dwarf plants indicated that shortening in the internodal length was associated with a smaller length in epidermal cells. Furthermore, transgenic plants expressing dwarf characters showed earlier flowering than normal one. The organ-specific transcription of the *rolC* gene was independent of the influence of other Ri TL-DNA genes.

1. INTRODUCTION

The soil bacterium *Agrobacterium rhizogenes* induces hairy roots when infecting most of dicotyledonous plants. Such a phenomenon is a consequence of the expression of Ri plasmid genes (Moor et al., 1979; White and Nester, 1980). Two transferred DNA (T-DNA) regions, designated TR- (the right T-DNA) and TL-DNA (the left T-DNA) of Ri plasmid A4, have been identified (Hoffman et al., 1984; Jouanin et al., 1984; De Paolis et al., 1985). The TR-DNA contains genes homologous to the *tms* region of the *A. tumefaciens* Ti plasmid (Boulanger et al., 1986), whereas the TL-DNA region does not (Slightom et al., 1986). The *tms* region encodes enzymes regulating auxin synthesis, by which hairy roots are induced (Cardarelli et al., 1987b). TL-DNA alone is also capable of inducing hairy roots (Cardarelli et al., 1987b; Vilaine et al. 1987a; Spanò et al., 1988). Nevertheless, the precise mechanism whereby genes in TL-DNA are responsible for root formation has not been elucidated.

Morphologically altered plants can be regenerated from Ri plasmid-induced hairy roots when cultured in a hormone-free medium (David et al., 1984; Tepfer, 1984). Plants possessing TL-DNA characteristically have wrinkled leaves, shortened internodes, rapid root growth, protruded pistils, reduced apical dominance, and other morphological changes (Tepfer, 1984; Pasqua et al., 1987; Nakamura et

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al., 1988). The entire nucleotide sequence of TL-DNA indicates the presence of 18 open reading frames (ORFs) (Slightom et al., 1986). Site directed mutagenesis experiments have shown that 4 loci of TL-DNA regulate hairy root formation, designated *rolA*, *B*, *C* and *D*, corresponding to ORF 10, 11, 12 and 15, respectively (White et al., 1985). The single locus (*rolA* or *rolB*) has the ability to trigger root formation in excised leaves or stems of tobacco, whereas *rolC* does not (Cardarelli et al., 1987a; Vilaine et al., 1987b; Spena et al., 1987).

Organ related expression of the *rolC* mRNA in transgenic tobacco plants possessing TL-DNA was reported to be high in roots and stems, but low in leaves (Schmülling et al., 1988; Nakamura et al., 1988). Furthermore, transgenic tobacco plants containing the *rolC* gene showed dwarfness, lack of apical dominance and other morphological changes (Oono et al., 1987; Schmülling et al., 1988). In this communication, we present early flowering, stable inheritance of dwarfism, morphological changes of floral organs and cell size of stem tissues in transgenic tobacco plants containing the *rolC* locus.

2. MATERIALS AND METHODS

Plant materials and analysis of growth and morphology

In a previous paper (Oono et al., 1987), we reported production of several transgenic plants of tobacco (T_0 generation) possessing the *rolC* gene [DNA fragment (*Hind*III-*Eco*RI; 1.9 kb) of Ri (A4) plasmid TL-DNA containing ORF12] by *Agrobacterium* mediated transformation system. Seeds (T_1 generation) obtained by self-crossing T_0 transgenic plants were germinated on a medium containing the Murashige and Skoog salts (Murashige and Skoog, 1962) with 0.8% agar and 300 mg/l kanamycin sulfate. Kanamycin resistant seedlings were selected and transferred to soil in a pot. Furthermore, the T_2 generation containing the homozygous *rolC* gene were also obtained from one of the self-crossed T_1 plants. Plants were allowed to grow until maturity under a 12 hr light (about 10,000 lux) at 26°C. An analysis of the variance of the size of floral organs was carried out according to the studentised range test (Snedecor and Cochran, 1967).

Histological analysis

The upper portion of stems of *rolC* transformants and Bin19 transformants were used for anatomical observation. Tissues were embedded in paraffin fixation in a formalin/acetic acid/chromic acid/dehydration procedure in tertiary butyl alcohol. About 15 μ m sections of paraffin embedded materials were stained with hematoxylin-eosine. The length and width of the cells of different tissues (epidermis, cortex, xylem and pith) were measured. Tests of significance of the cell size of stem sections were also carried out (Snedecor and Cochran, 1967). Logarithmically transformed crude data was analyzed.

mRNA analysis

RNA was extracted by guanidine thiocyanate according to the method of Chrigwin et al. (1979). Total RNA was run by the 1% agarose gel electrophoresis, followed by blotting to GENE SCREEN membrane (NEN Co. LTD.) according to the supplier's protocol. Probe DNA was labeled with [32 P]dCTP using the Multiprime DNA labeling system (Amersham).

3. RESULTS

Dwarfism in progeny plants

Seeds (T_1 generation) from self-crossed *rolC* transformants (Oono et al., 1987) were germinated on the medium containing kanamycin sulfate. After two weeks, seedlings with green (resistant) or white (sensitive) leaves segregated into a 3:1 ratio in all transgenic lines. Progeny plants of one of the transformants were then grown for a further period in soil, and several morphological characters were compared. The result of the analysis of variance indicates that progeny plants (T_2 generation) of the *rolC* transformant showed a shorter plant height than control (Table 1).

Table 1. Analysis of variance for the plant height of the transformed and non-transformed tobacco plants

Plant line	Mean of plant height (cm)	Degree of freedom	Mean square	F value
SR1	49.3 ^a	2	33800	118.94*
Bin19	48.3 ^a			
<i>rolC</i>	31.9 ^b			
Error			32	29

SR1: Non-transformed control (*Nicotiana tabacum* cv. Petite Havana SR1).

Bin19: Control Plants possessing the kanamycin resistant gene.

rolC: Transformed plants possessing both the kanamycin resistant gene and the *rolC* gene. T_2 generation (at least 10 plants for each lines) of Bin19 and *rolC* plants were used.

a, b: The mean values of plant height bearing the same letter are not significantly different at 1% level [$D(0.05)=5.46$ cm].

* Significantly different at 1% level.

Early flowering associated with dwarfism

Plants belonging to T_1 generation possessing the *rolC* gene were grown under the conditions described in the MATERIALS AND METHODS. A correlation was observed between the day-to-flowering and the final plant height. The plants having small, pistil-protruded-flowers were likely to show rapid flowering than

normal plants.

On the basis of this observation, we analyzed the dates required for flowering using individuals in T₂ generation which possessed the homozygous *rolC* gene. The results indicated that the average flowering days for the *rolC* transformants, the Bin19 transformants and the non-transformed control plants (SR1), were 119, 136 and 148 days, respectively (Table 2, Fig. 1-a). The analysis of variance in each line indicated significant differences (5% level) between *rolC* plants and the

Table 2. Analysis of variance for the flowering day of the transformed and non-transformed tobacco plants (T₂ generation)

Plant line	Mean of flowering days	Degree of freedom	Mean square	F value
SR1	148 ^a	2	11230	54.20*
Bin19	136 ^a			
<i>rolC</i>	119 ^b			
Error			32	29

a, b: The mean flowering days bearing the same letter are not significantly different at 5% level [D(0.05)=14.71].

* Significantly different at 5% level. At least 10 plants each of T₂ generation (Bin19 and *rolC*) were used.

Table 3. Analysis of the variance for the floral organs of the transformed and non-transformed tobacco plants

(a)

Factor	Degree of freedom	Mean square	F value
Plant lines (A)	2	9.2225	287.904*
Floral organs (B)	3	17.3391	541.281*
A × B	6	0.1252	3.910*
Error	44	0.0320	

* Significantly different at 1% level.

(b)

Plant line	Calyx	Corolla	Stamen	Pistil
SR1	15.9 ^a	41.1 ^d	42.3 ^d	42.1 ^d
Bin19	14.8 ^a	41.1 ^d	41.6 ^d	41.7 ^d
<i>rolC</i>	12.6 ^a	32.3 ^b	32.8 ^b	36.7 ^c

Numerals indicate mean length (mm) of each organ. Values bearing the same letters are not significantly different at 5% level [D(0.05)=4.2 mm]. The details are the same as described in the legend to Table 1.

Fig. 1. (a) An example of early flowering in the progeny plants (T_2 generation) possessing the dominant *rolC* gene (front line) and non-transformed control tobacco (back line). Floral organs of (b) a Bin19 transformant and (c) a *rolC* transformant. Stems of (d) a Bin19 transformant, and (e) a *rolC* transformant at early flowering stage. Arrows indicate lateral buds.

others. In the case of flower morphology, floral organs of the *rolC* progenies (T_2 generation) showed reduction in their lengths (Table 3). Unlike Bin19 plants (transformed by a vector Bin19 lacking the *rolC* gene), *rolC* transformants showed protruded pistils over stamens and corolla. (Fig. 1–b, c) The results obtained by the analysis of variance support this conclusion. It was noted that transgenic plants possessing the *rolC* gene showed a high incidence of pollen abortion. Such a partial male sterility was also inherited by progeny plants. Reduction of apical dominance observed in T_0 generation of *rolC* plants was also seen in the following T_1 and T_2 generations (Fig. 1–d, e).

Differences in cell size of stem tissues

Results of the cell size measurement in different tissues of the upper portion of the stem are shown in Table 4. Significant differences (5% level) in terms of the cell length are seen in epidermal cells when compared with control plants. The width of cells in tissues (pith) was also reduced in the transformants.

Table 4. Comparison of cell size (μm) of stem tissues in tobacco plants obtained by Bin19 and *rolC* transformants

Tissue		Bin19	<i>rolC</i>
Epidermis	(L)	134 ^f	81 ^e
	(W)	23 ^{bc}	28 ^{bc}
Cortex	(L)	147 ^f	130 ^f
	(W)	43 ^d	33 ^{cd}
Xylem	(L)	171 ^f	168 ^f
	(W)	15 ^a	21 ^{ab}
Pith	(L)	266 ^g	253 ^g
	(W)	73 ^e	28 ^{bc}

Values bearing the same letter are not significantly different at 5% level [D(0.05) calculated by logarithmically transformed data]. L, length of cells; W, width of cells. Mean values of 40 cells from different upper stem of two plants were recorded. T_0 generation was analyzed. The other details are the same as in the legend to Table 1.

Expression of rolC mRNA

The result of analysis of the *rolC* specific transcript in transgenic plants is presented in Fig. 2. As apparently seen here, the level of *rolC* mRNA is in the order of roots > stem $\approx$ flowers > leaves.

Fig. 2. Expression of the *rolC* mRNA in different organs of the *rolC* transformant. L: leaf. S: stem. R: root. F: flower. 25S, 18S: rRNA markers.

4. DISCUSSION

Although several experiments have been carried out to understand the function of the *rolC* gene, its product has not been identified. Sinkar et al. (1988) showed that regenerated plants from *rolC*⁻ hairy roots partially reverted to the normal plant height. They also demonstrated that *rolA*⁻ plants did not display the aberrant phenotype as seen in plants containing TL-DNA. Furthermore, *rolB*⁻ and *rolD*⁻ plants reduced the aberrant phenotype. By introducing *rolA* gene alone into tobacco plants, Sinkar et al. (1988) demonstrated that *rolA* is the primary determinant of the severely wrinkled phenotype of *Ri* plasmid transgenic plants.

Previously, we reported that the *rolC* gene of *Ri* plasmid (A4) was responsible for dwarfness in tobacco plants and reduced apical dominance (Oono et al., 1987). Similar results were also obtained by Schmülling et al. (1988). In the present study, we investigated several characters caused by the *rolC* gene in the progeny of transgenic tobacco plants. The *rolC* locus was transferred into the tobacco genome with the kanamycin resistant gene in the same plasmid Bin19.

The progeny resistant to kanamycin also showed altered characters similar to the parents, indicating morphological changes caused by the *rolC* gene were inherited stably. The stable inheritance of plant dwarfness was also confirmed in

the T₂ progeny of *rolC* plants conferring the homozygous kanamycin resistant genotype.

When we analyzed the relationship between days to flowering and the final plant height, the plants expressing the dwarf character tended to show earlier flowering than the normal ones. Similar observation was also made in other transgenic tobacco lines (data not shown).

An analysis of the cell size in different tissues of *rolC* transformants indicated that reduction in the length of epidermal cells could be taken as one of the main reasons for this type of dwarf characters. This notion was in agreement with a similar finding in the transgenic plants possessing the entire TL-DNA of Ri plasmid (our unpublished data).

Of particular interest is the pattern of mRNA expression in different organs in *rolC* transformants. As we reported earlier (Nakamura et al., 1988), mRNA in transformed plants possessing Ri TL-DNA showed the expression level in the order of root > stem > leaves. Similarly, the *rolC* gene was expressed at a low level in leaves, but at a high level in roots or stems in transgenic plants possessing *rolA*, *B* and *C* (Schmülling et al., 1988). Our results also indicate that the plant containing the *rolC* gene alone also expresses mRNA in the same order. Therefore, the organ specific mRNA expression of *rolC* was independent of the influence of the other Ri TL-DNA gene products. This finding strongly suggests the lack of transcriptional regulation in the expression of the *rolC* gene by other genes in TL-DNA. The mRNA expression in flowers is reasonable because many changes were seen in floral organs. Regarding tissue specificity of this gene, the *rolC* promoter was expressed mainly in phloem cells throughout plant organs (Sugaya et al., 1989; Schmülling et al., 1989).

Physiological function of Ri TL-DNA products is now likely associated with some phytohormonal regulation in plant growth (Spanò et al., 1988; Shen et al., 1988). Medford et al. (1989) reported that transgenic tobacco plants possessing isopentenyl transferase gene of *Agrobacterium tumefaciens* Ti plasmid showed reduced plant height and apical dominance. However, such plants grew normally in terms of date requirement for flowering. Since *rolC* transformants showed inhibited cell elongation and reduced apical dominance, the *rolC* gene product may interfere endogenous auxin-cytokinin balance. At present, we lack information concerning the biochemical nature of the *rolC* gene products in relation to phytohormonal association. It remains to be seen whether the *rolC* gene product directly stimulates flowering processes, or it is a consequence of dwarfness in plant growth.

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REFERENCES

- BOULANGER, F., BERKALOFF, A. and RICHAUD, F. (1986) Identification of hairy root loci in the T-regions of *Agrobacterium rhizogenes* Ri plasmids. *Plant Mol. Biol.* **6**, 271–279.
- CARDARELLI, M., MARIOTTI, D., POMPONI, M., SPANÒ, L., CAPONE, I. and COSTANTINO, P. (1987a) *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. *Mol. Gen. Genet.* **209**, 475–480.
- CARDARELLI, M., SPANO, L., MARIOTTI, D., MAURO, M. L., VAN SLUYS M. A. and COSTANTINO, P. (1987b) The role of auxin in hairy root induction. *Mol. Gen. Genet.* **208**, 457–463.
- CHIRGWIN, J. M., PRZYBYLA, A. E., MACDONALD, J. and RUTTER, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.
- DAVID, C., CHILTON, M. D. and TEMPÉ, J. (1984) Conservation of T-DNA in plants regenerated from hairy root cultures. *BiolTechnology* **2**, 73–76.
- DE PAOLIS, A., MAURO, M. L., POMPONI, M., CARDARELLI, M., SPANÒ, L. and COSTANTINO, P. (1985) Localization of agropine-synthesizing functions in the TR-region of the root inducing plasmid of *Agrobacterium rhizogenes* 1855. *Plasmid* **13**, 1–7.
- HUFFMAN, G. A., WHITE, F. F., GORDON, M. P. and NESTER, E. W. (1984) Hairy-root-inducing plasmid: physical map and homology to tumor inducing plasmids. *J. Bacteriol.* **157**, 269–276.
- JOUANIN, L. (1984) Restriction map of an agropine-type Ri plasmid and its homologies with Ti plasmids. *Plasmid* **12**, 91–102.
- MEDFORD, J. I., HORGAN, R., EL-SAWI, Z. and KLEE, H. J. (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *The Plant Cell* **1**, 403–413.
- MOORE, L., WARREN, G. and STROBEL, G. (1979) Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. *Plasmid* **2**, 617–626.
- MURASHIGE, T. and SKOOG, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–496.
- NAKAMURA, T., HANDA, T., OONO, Y., KANAYA, K., MICHIKAWA, M. and UCHIMIYA, H. (1988) Organ-specific mRNA in transgenic tobacco plants possessing T-DNA of Ri plasmids. *Plant Science* **56**, 213–218.
- OOONO, Y., HANDA, T., KANAYA, K. and UCHIMIYA, H. (1987) The TL-DNA gene of Ri plasmids responsible for dwarfness of tobacco plants. *Jpn. J. Genet.* **62**, 501–505.
- PASQUA, G., MONACELLI, B., ALTAMURA, M. M. and CALZECCHI-ONESTI, B. (1987) Transformed phenotype and *in vitro* flower neoformation in tobacco hairy root regenerants. *J. Plant Physiol.* **130**, 221–231.
- SCHMÜLLING, T., SCHELL, J. and SPENA, A. (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO J.* **7**, 2621–2629.
- SCHMÜLLING, T., SCHELL, J. and SPENA, A. (1989) Promoters of the *rolA*, *B*, and *C* genes of *Agrobacterium Rhizogenes* are differentially regulated in transgenic plants. *The Plant Cell* **1**, 665–670.
- SHEN, W. H., PETIT, A., GUERN, J. and TEMPÉ, J. (1988) Hairy roots are more sensitive to auxin than normal roots. *Proc. Natl. Acad. Sci. USA* **85**, 3417–3421.
- SINKAR, V. P., PYTHOUD, F., WHITE, F. F., NESTER, E. W. and GORDON M. P. (1988) *rolA* locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants. *Genes and Development* **2**, 688–697.
- SLIGHTOM, J. L., DURAND-TARDIF, M., JOUANIN, L. and TEPFER, D. (1986) Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid: identification of open reading frames. *J. Biol. Chem.* **261**, 108–121.

- SNEDECOR, G. W. and COCHRAN, W. G. (1967) *Statistical methods*. The Iowa State University Press, Ames.
- SPANÒ, L., MARIOTTI, D., CARDARELLI, M., BRANCA, C. and COSTANTINO, P. (1988) Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. *Plant Physiol.* **87**, 479–483.
- SPENA, A., SCHMÜLLING, T., KONCZ, C. and SCHELL, J. (1987) Independent and synergistic activity of *rolA*, *B* and *C* loci in stimulating abnormal growth in plants. *EMBO J.* **6**, 3891–3899.
- SUGAYA, S., HAYAKAWA, K., HANDA, T. and UCHIMIYA, H. (1989) Cell-specific expression of the *rolC* gene of the TL-DNA of Ri plasmid in transgenic tobacco plants. *Plant Cell Physiol.* **30**, 649–653.
- TEPPER, D. (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. *Cell* **37**, 959–967.
- VILAINE, F. and CASSE-DELBART, F. (1987a) Independent induction of transformed roots by the TL and TR regions of the Ri plasmid of agropine type *Agrobacterium rhizogenes*. *Mol. Gen. Genet.* **206**, 17–23.
- VILAINE, F., CHARBONNIER, C. and CASSE-DELBART, F. (1987b) Further insight concerning the TL region of the Ri plasmid of *Agrobacterium rhizogenes* strain A4: Transfer of a 1.9 kb fragment is sufficient to induce transformed roots on tobacco leaf fragments. *Mol. Gen. Genet.* **210**, 111–115.
- WHITE, F. F. and NESTER, E. W. (1980) Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. *J. Bacteriol.* **141**, 1134–1141.
- WHITE, F. F., TAYLOR, B. H., HUFFMAN, G. A., GORDON, M. P. and NESTER, E. W. (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. *J. Bacteriol.* **164**, 33–44.