Modified Development in Transgenic Tobacco Plants Expressing a *rolA*::GUS Translational Fusion and Subcellular Localization of the Fusion Protein

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Accepted 6 June 1998.

The *rolA* gene is transferred naturally by *Agrobacterium rhizogenes* to the genome of host plants, where it induces dramatic changes in development of transformed plants, including dwarfism and leaf wrinkling. The predicted translation product of the *rolA* gene is a small (11.4 kDa), basic (pI = 11.2) protein, which has no clearly significant similarity to sequences in the data bases. We have introduced into the tobacco genome a gene encoding a *rolA*::GUS fusion protein. Expression of this gene led to synthesis of an RNA and a protein of expected size, and the transformed plants exhibited the dwarfism and leaf wrinkling typical of *rolA* plants, but to a lesser degree than plants transformed with the wild-type *rolA* gene. The distribution of β -glucuronidase (GUS) activity was compared in subcellular fractions of leaf extracts from plants expressing either the *rolA*::*gus* gene or a control *gus* construct. As expected, in the control plants, GUS activity was essentially cytosolic. In contrast, in plants expressing the *rolA*::*gus* gene the highest specific activity was associated with the plasmalemma fraction.

The *rol* (root locus) genes (White et al. 1985), which are transferred naturally by *Agrobacterium rhizogenes* from the Ri plasmid to the nuclear genome of host plants, have profound effects on plant development (Tepfer 1984). When transformed with the *rolA* gene of strains A4 or 2659, plants of several species display a characteristic set of morphological modifications, including pronounced dwarfism, leaf wrinkling, and reduced fertility (Sinkar et al. 1988; Schmülling et al. 1988; Serino et al. 1994). The function encoded by *rolA* is at this time unknown. The *rolA* protein is predicted from the sequence to be of low molecular mass (11.4 kDa) and high isoelectric point (11.2), which has led to the hypothesis that it could interact with nucleic acids, and might be involved in regulation of gene expression (Levesque et al. 1988).

In order to better understand the morphological modifications of *rolA* plants, certain aspects of the physiology of tobacco plants expressing *rolA* have been examined. (Martin-

Tanguy et al. 1990; Maurel et al. 1991; Vansuyt et al. 1992; Dehio et al. 1993; Prinsen et al. 1994; Ben-Hayyim et al. 1996). The physiological studies of *rolA* plants contribute to our understanding of the cascade of events responsible for the morphological modifications observed in *rolA* plants, but are of limited use in predicting the nature of the *rolA* gene product itself. However, determination of the subcellular localization of the *rolA* protein would constitute a first step in understanding its function, since a DNA-binding protein would be expected to be nuclear, one involved in regulating hormone metabolism would probably be cytosolic, and one involved in hormone sensitivity or in transduction of hormone signals could be expected to be membrane localized. We have introduced into tobacco a gene encoding a bifunctional translational fusion between *rolA* and the *Escherichia coli* β -glucuronidase (GUS), and present here studies of the expression of the fusion gene and the localization of the fusion-protein gene product.

RESULTS

Construction of the *rolA*::*gus* gene and its transfer to the tobacco genome.

The four predicted *rolA* protein sequences were aligned to facilitate identification of conserved features (Fig. 1). The N-terminal half of the predicted *rolA* protein is highly conserved among the four strains, whereas the C-terminal half of the A4 *rolA* protein diverges from the other three. Examination of the A4 *rolA* nucleotide sequence, as confirmed by two different groups (Slightom et al. 1986; Sinkar et al. 1988), shows that this sequence divergence is due to a frameshift caused by a single nucleotide insertion/deletion in the A4 sequence after the codon of the serine residue at position 64, while the nucleotide sequence is equally well conserved over the entire *rolA* coding region (not shown).

The most striking conserved feature of the *rolA* protein sequences is the highly basic domain (residues 27 to 38). This domain is essential for *rolA* function, since plants do not display the *rolA* phenotype when transformed with *rolA* genes in which mutations affect either the Arg at position 38 or the Pro at position 41 (Dehio and Schell 1993). If the *rolA* protein is nuclear, then the basic domain could also contribute to its transport to this compartment (Raikhel 1992).

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For two reasons, we chose to construct a gene that would fuse the C terminus of the *rolA* peptide to the N terminus of the GUS protein, rather than the inverse. The above sequence analysis showed that the C-terminal portion of *rolA* is less conserved, and it has been shown that fusion of certain nuclear localizing signals to the N terminus of GUS is more efficient for nuclear transport than the corresponding C-terminal fusion (Raikhel 1992). Tobacco was transformed by standard protocols, and individuals expressing the *rolA::gus* gene were selfed in two successive generations to yield the homozygous plants used in further studies. Plant lines transformed with the *rolA::gus* gene were designated RAG(n), where n is the line number.

	10	20	30	40	50	
1724	MELAGINVVVG	MAQTFGEASL	VVSDLFRRAK	VKKKKAKRVS	PGDFLFDQIA	
2659	MELAGINVVVG	MAQTFGEASL	VVSDLFRRAK	VKKKKAKRVS	PGDFLFDQIA	
8196	MELAGINVAG	MAQTFGEVSL	VLSDLVRRAK	AKKKAKRVS	PGDVPPDQVA	
A4	MELAGLNVAG	MAQTFGVLSL	VCSKLVRRAK	A.KKKAKRVS	PGE...RDHLA	
cons	MELAG NV G	MAQTFG SL	V S L RRAK	K KAKRVS PG	D A	
			***	***		
	60	70	80	90	100	
1724	ELDDLSTVTP	AITSPGPTVM	VNDHGIATRG	RFVAGPEY..	SD LRG	
2659	ELDDLSTVTP	AITSPGPTVM	VNDHGIATRG	RFVAGPEY..	SD LRG	
8196	ELDDLSTVTP	AVTSPGPSVM	VNDHRIATRG	RFVGGPKN..	SN LRD	
A4	EPANLSTVTP	AMTSQARPRG	STTRELLRRD	PLSPDVKIQT	YGINTHFETN	LRD
cons	E LS TPL A TS		R		LR	
		↑	*		↑	*
		frameshift			GUS fusion	

Fig. 1. Comparison of *rolA* protein sequences. Peptide sequences were deduced from nucleotide sequences of the *rolA* genes of *Agrobacterium rhizogenes* strains 1724, 2659, 8196, and A4. Below the alignment is shown a consensus sequence (residues identical in all four proteins); positions where basic residues are conserved, indicated with asterisks. Position of a single nucleotide insertion/deletion leading to a frameshift in the gene of strain A4 and position of translational fusion with the *gus* coding sequence in the *rolA::gus* gene, shown by arrows.

Expression of the *rolA::gus* gene.

As shown in Figure 2, the phenotype of the resulting *rolA::gus* plants was similar to that of a reference tobacco line (Carneiro and Vilaine 1993) expressing the native *rolA* gene, *rolA*(3), although the leaf wrinkling and stem shortening were less intense than in the *rolA* plants. When total RNA from *rolA::gus* and *rolA* plants was probed with a *rolA*-specific probe, a transcript of expected size was observed (Fig. 3A). Similarly, when a similar blot was probed with a GUS-specific probe a single band of expected size was observed (not shown). When electropherograms of total protein of *rolA::gus* plants and of plants expressing a *35S-gus* gene were stained for GUS activity, the GUS-positive band in the *rolA::gus* plants was of higher molecular mass than that in *35S-gus* plants (Fig. 3B).

When GUS activity was compared among five *rolA::gus* lines, there was a correlation between the level of this activity and the intensity of the phenotypic modifications (not shown). These results suggest that the *rolA::GUS* fusion protein is bi-functional, since plants expressing the *rolA::gus* gene displayed the typical *rolA* phenotype, and the RNA and protein observed corresponded to the size expected for the fusion.

Subcellular localization of the *rolA::GUS* fusion protein.

We compared the localization of GUS activity in several lines expressing the *rolA::gus* gene and one expressing a control *35S-gus* gene. Figure 4A shows that, in *35S-gus* plants, the highest GUS activity was found in the cytosol, with progressively lower GUS activities in the successively heavier fractions (cytosol > microsomes > mitochondria > chloroplasts > nuclei). In contrast, the highest GUS activity was observed in the microsome fraction of all the *rolA::gus* lines tested. In particular, in RAG(6) plants, the microsomal GUS activity was five times that of the cytosolic activity. During

Fig. 2. Phenotype of tobacco plants expressing *rolA* and *rolA::gus* genes. Two-month-old homozygous plants of R2 generation are shown that express (left) native *rolA* gene (line *rolA*(3)), (center) *rolA::gus* gene (line RAG(3)), and (right) control, nontransformed tobacco.

the steps of Percoll purification, the GUS activity of the nuclear fraction progressively decreased (not shown), which indicated that the nuclear GUS activity observed was due to contamination by non-nuclear material.

After fractionation of microsomes by phase partitioning, the different subcellular fractions of 35S-*gus* plants had similar levels of GUS activity; the membrane-associated activity could be due to cytosolic proteins entrapped within the vesicles, and/or to GUS protein weakly associated with the different membrane fractions (Fig. 4B). In contrast, all the *rolA::gus* lines had much higher GUS activity in the plasmalemma fraction than in the other fractions tested. For example, plasmalemma vesicles of RAG(1) plants had 15- and 42-fold higher GUS activity than the microsome and total protein fractions, respectively.

Fig. 3. Expression of *rolA::gus* gene in tobacco. **A**, Northern (RNA) blot of total leaf RNA (30 µg per sample) probed with *rolA* coding sequence: control plant, *rolA*(3) plant, and RAG(3) plant. **B**, Visualization of β -glucuronidase (GUS) activity by polyacrylamide gel electrophoresis of total leaf protein (0.05 mg per sample): 35S-*gus* plant (GUS), RAG(3) plant, purified *Escherichia coli* GUS (Sigma, St. Quentin Fallavier, France) (GLU), and control plant (lane 4). Position of molecular mass markers is shown.

DISCUSSION

Transgenic tobacco plants expressing a *rolA::gus* translational fusion display the typical *rolA* phenotype. The results presented here clearly show that the *rolA* moiety of the *rolA::GUS* fusion protein strongly redirects GUS activity to the plasmalemma fraction, suggesting that the *rolA* protein itself is plasmalemma-associated. In contrast, it has been shown that another protein encoded by an *A. rhizogenes* gene, *rolD*, is cytosolic (Estruch et al. 1991). Thus, as we have shown that *rolA::GUS* is not nuclear, these results are not compatible with the hypothesis that *rolA* has a direct role in regulation of gene expression via protein-DNA interactions (Levesque et al. 1988). Since the *rolA::GUS* protein is plasmalemma-associated, the *rolA* sequence was further examined for potential structures that could be responsible for this localization. The N-terminal 25 residues are rich in hydrophobic amino acids (Fig. 1). However, since this hy-

Fig. 4. β -Glucuronidase (GUS)-specific activity in subcellular fractions. GUS activity was determined in leaf extracts that had been separated into fractions enriched in (A) nuclei, chloroplasts, mitochondria, microsomes, and cytosol, and (B) total soluble protein, microsomes, plasmalemma, and intracellular microsomal membranes. Bars show mean values $\pm$ SE obtained in three independent experiments.

drophobic domain is interrupted by hydrophilic residues (Asn and Gln in the A4 *rolA* protein, with additional Asp and Glu in *rolA* of the other strains), it is unlikely that this domain could form a transmembrane segment. Likewise, the *rolA* proteins lack potential amphiphilic helices or sites of attachment of lipid anchors (not shown). The absence of what could clearly be transmembrane domains in the *rolA* protein is consistent with *rolA::GUS* being a non-integral, membrane-associated protein.

MATERIALS AND METHODS

Sequence analysis of the *rolA* gene and its translation product.

The sequences analyzed correspond to the *rolA* gene from *A. rhizogenes* strain A4 (accession numbers K03313 [Slightom et al. 1986] and X12579 [Sinkar et al. 1988]), strain 8196 (accession number M60490 [Hansen et al. 1991]), strain 2659 (accession number Z29365 [Serino et al. 1994]), and strain 1724 (accession number X64255 [Kiyokawa et al. 1994]). Sequence similarity was studied with various programs (BLAST, Pileup, Pretty, etc.) of the University of Wisconsin Genetics Computer Group (GCG) sequence analysis package (Devereux 1991).

Construction of the *rolA::gus* gene and its transfer to the tobacco genome.

A plasmid bearing the entire *rolA* gene in a 1.9-kb *EcoRI*-*NruI* fragment (pRA1) was cleaved with *NcoI* and submitted to exonuclease Bal31 hydrolysis, after which fragments were rendered blunt-ended by Klenow polymerase treatment. The fragment containing the *rolA* gene was then isolated by *XhoI* hydrolysis, and cloned into pBS-KS⁺ digested with *SmaI* and *XhoI*. A clone bearing the *rolA* open reading frame minus the last 15 nucleotides was selected. An *XhoI*-*BamHI* fragment containing the entire truncated gene was inserted into the plasmid pBRA1GUS (Carneiro and Vilaine 1993), replacing the *XhoI*-*BamHI* fragment bearing the *rolA* promoter, giving the plasmid proLAGUS, which was introduced into *Agrobacterium tumefaciens* strain GV3101(pMP90) (Koncz and Schell 1986) according to the method described by Zham et al. (1984). Transformed *Nicotiana tabacum* cv. Xanthi XHFD8 (Bourgin 1978) was obtained with the resulting *Agrobacterium* strain according to Horsch et al. (1985). The 35S-*gus* controls were obtained by transforming the same tobacco genotype with *Agrobacterium* bearing pBI121 (Jefferson 1987).

Expression of the *rolA::gus* gene.

Total RNA was extracted from leaves of 2-month-old, greenhouse-grown tobacco plants, according to the method described by Verwoed et al. (1989). Thirty micrograms of total RNA from each plant was submitted to electrophoresis in a denaturing agarose gel (Lehrach et al. 1977). After transfer to a nylon membrane (Pall Biodyne B) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), hybridization was performed at 50°C in the buffer of Church and Gilbert (1984). DNA corresponding precisely to the A4 *rolA* open reading frame was amplified from pRA1 by polymerase chain reaction, ³²P-labeled by random priming, and used as probe (Feinberg and Vogelstein 1983).

Subcellular localization of the *rolA::GUS* fusion protein.

Differential centrifugation and Percoll purification were employed to separate homogenates into fractions enriched in chloroplasts, mitochondria, microsomes, and cytosol (Rembur et al. 1983). Intact nuclei were purified from mesophyll protoplasts according to Saxena et al. (1985). Plasmalemma was extracted from the 12,000 to 40,000 g of microsomal fraction by the aqueous, two-polymer, phase-partitioning method (Lundborg et al. 1981), with a final composition of 6.2% (wt/wt) Dextran T500 (Pharmacia, Orsay, France) and 6.2% (wt/wt) polyethylene glycol 3350 (Sigma, St. Quentin Fallavier, France). Phase partitioning was conducted three times. Purity of the plasmalemma fraction obtained by phase partitioning was checked by measurements of ATPase activity at pH 6.5 (Perlin and Spanswick 1980). Measurements were carried out in the presence of various specific inhibitors according to Rembur et al. (1986). ATPase activity was inhibited 90% by 200 μM orthovanadate, a specific inhibitor of plasmalemma ATPase. In contrast, the addition of 100 μM molybdate (specific inhibitor of tonoplast ATPase) or 0.5 μg of oligomycin (inhibitor of mitochondrial ATPase) per ml reduced activity only weakly (5 to 10%). Absence of chlorophyll was shown according to Arnon (1949). These results show that there was very little contamination of the plasmalemma fraction.

GUS activity of fractions was determined with the fluorogenic substrate, 4-methylumbelliferyl glucuronide (MUG) according to Jefferson (1987). Protein content was determined by a modification of the Bradford method (Bio-Rad Laboratories, Ivry sur Seine, France), with bovine gamma globulin as standard. Data are expressed as picomoles of 4-MU liberated per minute per mg of protein.

Protein samples were analyzed on a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) minigel (Laemmli 1970) after incubation at 40°C for 1 min. Gels were washed in extraction buffer (Jefferson 1987), incubated in fresh buffer containing 0.5 mM MUG for 20 min, and rinsed in 0.2 M Na₂CO₃. Gels were photographed on a 312 nm transilluminator with Polaroid type 665 film.

ACKNOWLEDGMENTS

This research was supported in part by grant number 92.T.0355 from the French Ministry of Research.

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