Promoters of the rolA, B, and C Genes of Agrobacterium rhizogenes Are Differentially Regulated in Transgenic Plants

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Chimeric genes containing the β-glucuronidase reporter gene under the control of the rolA, B, and C promoters of Agrobacterium rhizogenes are expressed in a regulated manner in transgenic plants. The intergenic region separating the rolB and C genes represents a bidirectional promoter. This bidirectional promoter regulates transcription for both genes in a similar fashion in aerial organs of the plants, but in a distinct way in roots. Moreover, both rolB and C promoter activities differ from those characteristic of the rolA promoter. Thus, promoters of bacterial origin show differential expression in transgenic plants, and regulation of rol gene expression plays a role in the biological effects caused by the rolA, B, and C genes.

INTRODUCTION

The rolA, B, and C genes of Agrobacterium rhizogenes are located on the TL-DNA of Ri plasmid A4 and are involved in the pathogenesis of the hairy-root disease (White et al., 1985; Cardarelli et al., 1987; Spena et al., 1987; Vilaine, Charbonnier, and Casse-Delbart, 1987). When expressed separately in transgenic tobacco plants, they establish phenotypic alterations characteristic for each gene (Oono et al., 1987; Schmülling, Schell, and Spena, 1988; Sinkar et al., 1988). Deregulation of expression of the rolB and C genes by substituting their own 5'-flanking regions with the 35s promoter from cauliflower mosaic virus generates new and distinct developmental alterations in transgenic plants, indicating that regulation of rol gene expression plays a relevant role in determining the type of morphological alterations observed in transgenic plants regenerating from hairy roots (Schmülling, Schell, and Spena, 1988). RNA gel blot analysis performed with poly(A+) RNA from different organs of plants regenerated from Ri-induced roots (Schmülling, Schell, and Spena, 1988) had indicated that the rolB and C genes were expressed in an organ-specific manner.

The rolA, B, and C genes correspond to open reading frames 10, 11, and 12, respectively, of the TL-DNA of Ri plasmid A4, as determined by Slightom et al. (1986). We have investigated rol promoter activities by positioning the β-glucuronidase reporter gene (Jefferson, Kavanagh, and Bevan, 1987) under the control of the three rol 5'-flanking sequences in order to analyze their organ and cell specificity of expression in transgenic plants. We present data showing that the rolB and C bidirectional promoter, which is located on the intergenic region between the rolB and C genes, has distinct promoter activities, and that rolA promoter activity differs from that of both rolB and C.

RESULTS

Analysis of rolA, B, and C Promoter Activities in Transgenic Plants by Fluorometric Assays

Chimeric genes were constructed by positioning the β-glucuronidase (GUS) reporter gene under the control of the rolA, B, and C 5'-flanking regions (Figure 1). The different constructions were subcloned in the binary vector pPCV002 (Koncz and Schell, 1986) and delivered to plant cells via Agrobacterium-mediated transformation. Transgenic plants were regenerated by standard techniques (Horsch et al., 1984, 1985), and fluorometric assays for β-glucuronidase were performed as described (Jefferson, 1987). In different clones, probably due to position effects, the variability in β-glucuronidase activity was in the range of 150-fold when measured using callus material. The values given in Table 1 were obtained by measuring β-glucuronidase activity in high expressing plants. The results show that the rolA, B, and C 5'-flanking sequences establish different levels of GUS activity in roots, stems, and leaves, and thus confirm previous data obtained through RNA gel blot analysis (Durand-Tardif et al., 1985; Ooms et al., 1986; Spena et al., 1987; Schmülling, Schell,
and Spena, 1988). Whereas rolB promoter activity in old leaves is 50-fold lower than in stems, rolC and rolA expression is only reduced approximately 10 times. In young leaves of both AGUS and BGUS1177 transgenic plants, GUS activity is 5 times lower than in stems, whereas in CGUS plants, GUS activity is only halved. Moreover, reducing the rolB promoter to only 305 bp (pBGUS305) weakens the intensity of expression. This result is in agreement with the curtailed capacity to induce root formation shown by the rolB gene when expressed under the control of only 305 bp of its 5'-flanking region (Spena et al., 1987).

Visualization of Organ and Cell Specificity of the rolA, B, and C Promoters by Histochemical Stainings

Expression of β-glucuronidase in transgenic plants allows one to perform histochemical stainings of plant sections and of whole plant organs (Jefferson, 1987; Jefferson, Kavanagh, and Bevan, 1987). Histochemical stainings of leaves transgenic for the different GUS constructions (Figure 1) show that all three promoter activities are detected mainly in phloem cells (Figures 2A and 2B). Stem sections from plants transgenic for AGUS, BGUS (data not shown), and CGUS (Figure 2C) localize promoter activity to the external and internal phloem cells (Esau, 1965). Similarly, Figure 2D presents a histochemical staining of anthers transgenic for the CGUS chimeric gene, showing that β-glucuronidase activity is localized mainly in the vascular tissue. Figure 2E shows a section of the shoot apex of a BGUS transgenic plant stained in the vascular tissue but not in the apical meristem.

Roots transformed with the BGUS (Figures 3A, 3B, and 3C) and CGUS chimeric genes (Figures 3D and 3E) show different patterns of expression. Figure 3A shows that BGUS expression is specifically detectable in the root cap and in the apical part of the region of cell division, whereas along the main root, regions of GUS activity correspond to the organization and emergence of side roots (Figures 3B and 3C). This feature is specific for the BGUS gene and was not observed in roots transgenic for CGUS. Expression of the CGUS chimeric gene is phloem-specific (Figures 3D and 3E) and detectable in both the main and the side roots. Therefore, the rolB and C promoter activities differ in their patterns of expression within roots.

The evaluation of AGUS gene expression in roots revealed a pattern of expression different from those observed in roots transgenic for BGUS and CGUS chimeric genes. Indeed, Figure 3F shows that AGUS gene expression is not detectable in emerging side roots.

| Table 1. β-Glucuronidase Activity in Stems, Roots, and Leaves of Transgenic Tobacco |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Construction                    | Plants Tested | Stems Roots Leavesa | Old Leavesb |
| pAGUS671                        | 7              | 450 280 90       | 40              |
| pBGUS305                        | 7              | 40 10 2          | 1               |
| pBGUS1177a                      | 14             | 150 30 30        | 3               |
| pCGUS1181                       | 7              | 1,600 830 850    | 200             |
| p3SSGUS                         | 7              | 11,500 9,600 8,400 | 9,000         |

Activities were expressed in picomoles of the reaction product methylumbelliferone per milligram of protein per minute. Extracts from SR1 untransformed control plants gave values less than 0.1 pmol of methylumbelliferone/mg of protein x min^-1. Data were obtained from regenerant plants 4 weeks after subculturing. The mean standard deviations of the different measurements ranged between 14% and 35%, with the exception of stems of plants transgenic for the BGUS305 and BGUS1177 reporter genes, where the standard deviations were 61% and 46%, respectively.

a Young leaves were less than 1 cm long.

b Old leaves were taken from the basal and middle part of the plants.

Plants transgenic for BGUS1177 gene inserted in both orientations were tested, and no difference in the pattern of expression was observed.
Figure 2. Histochemical Stainings for GUS Activity in Leaves and Stems.

(A) Histochemical staining for GUS activity in leaves of a CGUS transgenic plant.
(B) Higher magnification of stained leaf tissue from a CGUS transgenic plant after destaining endogenous pigments with commercial bleach. CGUS activity is localized mainly to cells of the vascular system.
(C) Stem section from a plant transgenic for the CGUS gene showing localization of GUS activity in the external and internal phloem cells.
(D) Anther section of a CGUS transgenic plant after staining for GUS activity. In this organ also, rolC promoter activity is restricted mainly to phloem cells.
(E) Longitudinal section from the apical part of a BGUS transgenic plant after staining for GUS activity. Promoter activity is not detectable in the apical meristem.
Figure 3. Histochemical Stainings for GUS Activity in Roots.

(A) Staining for GUS activity in roots expressing the BGUS chimeric gene. Promoter activity is localized mainly in the root cap and in the region of cell division.

(B) Staining for GUS activity of roots expressing the BGUS chimeric gene. Promoter activity is localized mainly in the root primordia.

(C) Staining for GUS activity of roots expressing the BGUS chimeric gene. Promoter activity is localized mainly in the root primordia.

(D) Staining for GUS activity of roots expressing the CGUS chimeric gene. In roots also, the rolC promoter activities are localized mainly to the phloem.

(E) Cross-section of roots expressing the CGUS chimeric gene. Promoter activity is localized mainly to the phloem.

(F) AGUS transgenic root stained for GUS activity. In contrast to the rolB and rolC promoter activities, the rolA promoter is not active in emerging side roots.
DISCUSSION

The rol genes of A. rhizogenes Ri plasmid A4 are involved in the pathogenesis of the hairy-root disease (White et al., 1985) and in the establishment in transgenic plants of complex developmental and morphological alterations (Cardarelli et al., 1987; Jouanin et al., 1987; Spena et al., 1987). When expressed individually, the rolA, B, and C genes induce root formation in transformed plant cells and plant tissues (Cardarelli et al., 1987; Spena et al., 1987; Vilaine, Charbonnier, and Casse-Delbart, 1987), and establish in transgenic plants developmental alterations characteristic of each type of gene (Oono et al., 1987; Schmülling, Schell, and Spena, 1988). Indeed, exchanging the rolC or the rolB 5′-flanking regions for the 35S promoter of cauliflower mosaic virus generates novel developmental alterations in transgenic plants (Schmülling, Schell, and Spena, 1988).

These new developmental alterations are due to a different organ and cell specificity of the rolB, rolC, and 35S promoters. Whereas expression of the rolB and C promoters in leaves is limited mainly to the vascular system (Figures 2A and 2B), activity of the 35S promoter is detected also in other cell types (data not shown; Jefferson, Kavanagh, and Bevan, 1987). Although both rolB and C genes are able to induce root formation (Cardarelli et al., 1987; Spena et al., 1987) and to influence root morphology and growth (White et al., 1985; Schmülling, Schell, and Spena, 1988), their specific effects on root initiation and growth are different (Schmülling, Schell, and Spena, 1988). Moreover, the rolB and C promoter activities differ in their patterns of expression in roots. The BGUS chimeric gene is expressed mainly in the root cap and in the region of cell division, and, at a much lower level, in the phloem, whereas CGUS expression occurs mainly in the phloem. Therefore, not only the synergistic activities of the rol gene products but also the different specificities of their bidirectional promoter might play a role in the pathogenesis of the hairy-root disease and in the establishment of the hairy-root syndrome in transgenic plants.

Based on the different biological effects on root formation and growth (i.e., root branching) of the rolB and C genes, it is conceivable that the different specificities displayed by their promoters allow a concerted action of the two rol genes at the whole root level. In this regard, it is interesting to note that White et al. (1985) reported that insertions between the rolB and C loci attenuated root formation, and the capacity of the rolB gene to induce roots is reduced when controlled by a truncated form of its promoter consisting of only 305 bp out of 1177 bp (Spena et al., 1987). In this article, we have reported that the truncated rolB promoter shows the same pattern of expression as the whole promoter, but its strength is reduced (Table 1). Thus, in the region between 305 bp and 1177 bp upstream of the initiation codon of the rolB gene, DNA sequences able to increase rolB promoter activity must be located. However, deregulated expression of the rolB gene also decreases its capacity to trigger root formation (Spena et al., 1987), suggesting that modulation of rolB gene expression plays a relevant role in maximizing root formation. Taken together, our results show that the rolA, B, and C promoters, as other T-DNA derived promoters (Koncz and Schell, 1986; An et al., 1988), establish a complex pattern of gene expression in transgenic plants.

METHODS

Bacterial Strains and Cultures

Bacterial strains and cultures were described previously (Spena et al., 1987).

Construction of Plasmids

Standard techniques were used for the construction of recombinant plasmids (Maniatis, Fritsch, and Sambrook, 1982), p35SGUS was built by inserting the 35SGUS chimeric gene (derived from RT99gus, Topfer, Schell, and Steinbiss, 1988) into the EcoRI site of the binary vector pPCV002 (Koncz and Schell, 1986). pBGUS1177 and pCGUS1181 contain the 1181 bp-long BamHl/Hpal fragment of the rolB/rolC intergenic region (Slightom et al., 1986) positioned in either orientation in front of the GUS coding region. Since the BamHl site is located 4 bp in the rolB coding region, the region tested for promoter activity in the BGUS1177 construction is 1177 bp long. In the CGUS1181 construct, the region tested for promoter activity includes the first 4 bp of the rolB coding region, and consequently is 1181 bp long. The chimeric BGUS and CGUS genes were subcloned in the EcoRI site of pPCV002. A Hindlll deletion of pBGUS1177 led to pBGUS305, where only 305 bp of the rolB 5′-flanking sequences control the expression of the reporter gene. In both cases (i.e., BGUS1177 and BGUS305), 7 amino acids were added at the NH2 terminus of the β-glucuronidase. A BamHl deletion, up to position +27 of the rolA coding region, was fused to the GUS gene present in plasmid pB101.2 (Jefferson, 1987), and then the chimeric gene was subcloned as an EcoRI/Hindlll fragment into the vector pPCV002, thus obtaining pAGUS671. Therefore, the AGUS gene product carries 18 additional amino acids at this NH2 terminus. All constructions were transferred to Escherichia coli strain SM10 and then mobilized to Agrobacterium tumefaciens strain GV3101 as described (Koncz and Schell, 1986).

Plant Tissue Culture and Transformation

Transgenic Nicotiana tabacum Petit Havana SR1 (Nagy and Maliga, 1976) were raised by following a modified leaf disc trans-
formation procedure (Horsch et al., 1984, 1985). Analysis of the progeny (obtained by self-pollination) was performed on three independent clones for each construct.

Fluorometric and Histochemical Analysis of GUS Activity

Extracts of 10 mg to 30 mg of plant tissue were incubated at 37°C in GUS extraction buffer (Jefferson, 1987), containing 1 mM 4-methylumbelliferyl glucuronide. The reaction was stopped after 15 min to 120 min, and the concentration of the reaction product (methylumbelliferone) was determined fluorometrically as described (Jefferson, 1987). Protein concentration of plant extracts was determined by the method of Bradford (1976). Hand-cut sections of transgenic plants were fixed and stained as described (Jefferson, 1987) using the indogenic substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide).

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REFERENCES


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