The role promoter of *Agrobacterium rhizogenes* Ri plasmid is activated by sucrose in transgenic tobacco plants

Abstract

The 5'-upstream region of the rolC gene of the Ri plasmid is expressed specifically in phloem cells of transgenic higher plants. In this study, we demonstrated that the rolC promoter is activated by sucrose in phyloem cells of transgenic tobacco seedlings bearing rolC promoter-uidA chimeric fusion gene. Since the rolC promoter is not activated by sorbitol, sucrose metabolism rather than osmotic pressure exerted by the disaccharide may be responsible for induction. Thus, experiments using 5'-upstream deletion mutants, internal deletion mutants, and chimeric constructs with a heterologous promoter (−90 region of the cauliflower mosaic virus 35S promoter) were conducted to define the region of the rolC promoter involved in sucrose activation. The results indicated that a cis-acting sucrose responsive region of the rolC promoter is located between −135 and −94 bp with respect to the transcription initiation site. In phloem cells, high concentrations of sucrose are encountered owing to ongoing translocation of photosynthates from source to sink tissues. Therefore, sucrose as a signal molecule may regulate the phloem-specific expression of the rolC promoter.

Key words rolC promoter • β-Glucuronidase • Cis-element • Sucrose induction

Introduction

The soil bacterium *Agrobacterium rhizogenes* harboring the Ri plasmid infects most dicotyledonous plants, resulting in production of adventitious roots (Moore et al. 1979; White and Nester 1980). Plants regenerated from such hairy root cultures possessing the T-DNA of the Ri plasmid show abnormal morphological characteristics such as wrinkled leaves, short internodes and reduced apical dominance (David et al. 1984; Tepfer 1984). Eighteen open reading frames (ORFs) are located in the TL-DNA of the Ri plasmid (Slightom et al. 1986). Previously, we demonstrated that tobacco plants possessing ORF12 (the rolC gene) also showed abnormal features such as dwarf morphology in plants (Oono et al. 1987) and early flowering (Oono et al. 1990). The transcriptional level of this gene varies among organs, the order being roots > stems > leaves (Nakamura et al. 1988). Using transgenic plants containing the rolC promoter-uidA (β-glucuronidase, GUS) fusion gene, evidence was obtained showing that the rolC promoter is expressed in specific cells, namely in the phloem cells of both dicots (Sugaya et al. 1989; Schmülling et al. 1989) and monocots (Matsuaki et al. 1989). Furthermore, we found that the expression of GUS directed by the rolC promoter was enhanced during somatic embryo development of carrots (Fuji and Uchimiya 1991). Deletion analysis of the rolC promoter indicated that a cis regulatory element responsible for phloem-specific expression was present within the 5’-upstream region (−153) of the rolC gene (Sugaya and Uchimiya 1992). We also showed that nuclear proteins prepared from tobacco hairy roots (Kanaya et al. 1990) and wheat germ (Kanaya et al. 1991) also bound to the −153 region.

We have been interested in finding the signals related to cell-specific and developmentally regulated expression of the rolC gene. Thus, we investigated the effects of various external stresses and molecules on the level
of GUS expression regulated by the rolC promoter. We found that a high concentration of sucrose activated expression of the GUS fusion gene in transgenic tobacco seedlings. Under such circumstances, activation of rolC-uidA was confined to phloem tissues through which sucrose is translocated from source tissues (leaves) to sink (roots). Sucrose applied exogenously to plant organs also activates genes, such as the class-I patatin gene (Wenzler et al. 1989; Rocha-Sosa et al. 1989), the proteinase inhibitor-II gene (Johnson and Ryan 1990), the sporamin gene (Hattori et al. 1991), the chalcone synthase gene (Takaya et al. 1991), and the vegetative storage protein acid phosphatase gene (Mason et al. 1993). In the case of the class-I patatin gene, a cis-acting sucrose-responsive region was defined, where AT-rich sequences were noted (Liu et al. 1990; Jefferson et al. 1990).

In this study, we assessed the conditions where high concentrations of sucrose activated the rolC-uidA fusion gene in phloem cells of transgenic tobacco seedlings. Furthermore, analysis of a cis-acting sucrose-responsive region of the rolC promoter is presented.

Materials and methods

Plasmid constructions

The plasmid pH101-012-uidA and the S′ deletions of the rolC promoter have been described in Sugaya and Uchimiya (1992). Internal deletions of the rolC promoter were made as follows. DNA fragments (blunt end-EcoRI) containing a series of deletion derivatives (−255, −230, −192, −135, and −94 bp) of the upstream region of the rolC gene and the coding region of the GUS gene were inserted into the Smal/EcoRI sites of pUC-848/−255 [pUC19 containing the rolC promoter (−848 to −255 bp) at the HindIII site]. DNA fragments (HindIII-EcoRI) deleted for regions upstream of the rolC promoter (Δ−255/−255, Δ−230, Δ−255/−192, Δ−255/−135, Δ−255/−94, see Fig. 7A) and the GUS gene were inserted into the HindIII/EcoRI sites of pB119.

The EcoRV-BamHI fragment containing the truncated (−90 to +6 bp) CaMV35S promoter and the BamHI-EcoRI fragment containing the GUS gene and nos terminator of pB1221 (Jefferson et al. 1987) were inserted into the HindIII/EcoRI sites of pH101, creating the CaMV35S truncated promoter-uidA fusion gene. The upstream region of rolC-uidA (to −255 bp) in pUC12 was digested with AsaII, end-filled with Klenow polymerase and subsequently digested with HindIII, creating the fragment of the rolC promoter region extending from −255 bp to −94 bp. The rolC promoter region from −848 to −255 bp was obtained by HindIII and SmaI digestion of pUC-848/−255. These fragments of the rolC promoter region (HindIII blunt end) were subcloned in the HindIII fills-in XhoI site of the CaMV 35S truncated promoter-uidA fusion gene in pB119. Subsequently, heterologous promoter-GUS gene (HindIII-EcoRI) was inserted into the HindIII/EcoRI sites of pB119.

Plant transformation

Leaf discs of Nicotiana tabacum cv. Petit Havana cv. SR1 were infected with Agrobacterium tumefaciens containing various deletion mutants. Transformed tissues were grown on Murashige–Skog (MS) medium (1962) containing 0.1 μg/ml naphthalene acetic acid (NAA), 1.0 μg/ml benzyladenine (BA), 500 μg/ml clafor, and 100 μg/ml kanamycin sulfate for shoot induction. Transgenic tobacco plants were further grown on the same medium devoid of phytohormones.

RNA isolation and RNA gel blot analysis

RNA was extracted from untreated and sucrose-treated transgenic tobacco seedlings (about 1g), using guanidium thiocyanate (Chirgwin et al. 1979). Electrophoresis of total RNA (20 μg) was in a 1.2 % agarose gel. The RNA was blotted onto nylon membranes and hybridized with a GUS-specific DNA probe labeled by the random priming method (Feinberg and Vogelstein 1983) in the presence of [α-32P]dATP (Amersham, 3000Ci/mmol). The mRNA levels were quantitated with a Bio-imaging analyzer (FUJIX BAS2000).

Plant materials

Transgenic tobacco seedlings bearing either rolC-GUS or various deletion mutants of rolC-GUS were grown on MS (Murashige and Skog 1962) agar medium lacking phytohormones and sugars under 10000 lux illumination (16 h day length). Seedlings (5 days old) were floated on liquid MS medium containing various supplements, but lacking phytohormones, for 48 h with gentle shaking. Experiments were repeated at least three times on R1 seedlings harvested from one independent transgenic tobacco plant.

Preparation of tissue extracts

Plant tissues (10 seedlings) were homogenized with a lysis buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% TritonX-100, 0.1% Sarkosyl and 10 mM 2-mercaptoethanol) and quartz sand in 1.5 ml Eppendorf tubes. Soluble protein fractions obtained by centrifugation (8500 g, 5 min) were used for GUS measurements (Jefferson et al. 1987) and quantitation of proteins (Bradford 1976).

GUS assay

Fluorometric quantitation of GUS was according to Jefferson et al. (1987). The supernatant fraction (80 μl) after centrifugation (8500 g, 5 min) was mixed with 170 μl lysis buffer and 250 μl 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG). The reaction was terminated by adding 2 ml 0.2 M Na2CO3 to 100 μl reaction mixture after 60 min incubation at 37 °C. Fluorescence emission at 455 nm was measured with excitation at 365 nm. GUS activity was expressed as pmoles 4-methylumbelliferosf (4 MU) per minute per milligram protein.

Histochemistry

Seeds of non-transgenic and transgenic tobacco N. tabacum cv. Samsun possessing a rolC promoter uidA chimeric gene (Sugaya et al. 1989) were sterilized with 5% (w/v) hypochloite for 20 min and germinated in MS solid medium (Murashige and Skog 1962) containing 50 mg/l kanamycin sulfate for resistance selection but lacking sucrose. Two-week-old seedlings were transferred in MS liquid medium containing 400 mM sucrose and incubated for 48 h with gentle agitation. Seedlings were sectioned cotyledonary leaf, hypocotyl and root) fixed in freshly prepared 10 mM MES, pH 7.0 containing 0.3% (w/v) formaldehyde and 0.3 M mannitol for 30 min. A brief vacuum (5 min) was employed to facilitate the penetration of the fixative. Tissues were washed with 50 mM sodium phosphate.