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Expression and Regulation of the *RSI-1* Gene during Lateral Root Initiation

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The tomato gene *RSI-1* was previously identified as a molecular marker for auxin-induced lateral root initiation. We have further characterized the expression mode of the *RSI-1* gene in tomato and *Arabidopsis thaliana*. Northern blot analyses revealed that the gene was induced specifically by auxin in tomato roots and hypocotyls. For experiments with transgenic plants, the 5' flanking region of the *RSI-1* gene was linked to a GUS reporter gene, then transformed into tomato and *Arabidopsis*. In these transgenic tomato plants, GUS activity was detected at the sites of initiation for lateral and adventitious roots. Expression of the fusion gene was auxin-dependent and tissue-specific. This was consistent with results from the northern blot analyses. In transgenic *Arabidopsis*, the overall expression pattern of the *RSI-GUS* gene, including tissue specificity and auxin inducibility, was comparable to that in transgenic tomato seedlings. These results indicate that an identical regulatory mechanism for lateral root initiation might be conserved in both plants. Thus, the expression mode of the *RSI-GUS* gene in *Arabidopsis* mutants defective in lateral root development should be investigated to provide details of this process.

Keywords: *Arabidopsis thaliana*, auxin, lateral root, tomato, transgenic plant

Plants increase their absorptive capacity by forming lateral roots that branch from a primary root. These lateral roots develop near the xylem poles of the vascular cylinder, some distance behind the root tip (Mauseth, 1988). Their development is initiated by cell division in the pericycle, the outermost single-cell layer at the periphery of the stele (Charlton, 1991).

In addition to lateral roots, adventitious roots may also arise on any organ that is not a primary root (Mauseth, 1988). Adventitious roots initiate in almost any tissue, including the epidermis, stem cortex, and stem pericycle of monocots and dicots. The initiation of lateral or adventitious roots is followed by the formation of a primordium that differentiates into a new meristem (Laskowski et al., 1995). This process provides an excellent model system for the study of root development.

The plant growth regulator, auxin, may be a signal for lateral root initiation. Exogenously applied auxin dramatically increased the frequency of lateral root formation in most dicot plants, including radish (Blakely et al., 1982, 1988), pea (Hinchee and Rost, 1986), lettuce (MacIsaac et al., 1989), and tomato (Taylor and El-Kheir, 1993). Overexpression of bacterial auxin biosynthetic genes in transgenic plants also resulted in increased lateral root formation (Klee et al., 1987; Kares et al., 1990). However, the molecular mechanisms associated with auxin-induced lateral root development are poorly understood.

Identifying the genes that are specifically expressed in lateral primordia is the initial step toward a detailed understanding of lateral root initiation mechanisms. Recently, a number of genes expressed in the very early stages of lateral root development were isolated from tomato. The gene, *RSI-1* (*Root System Inducible-1*), was detected in a small subset of pericycle cells just prior to undergoing a first round of cell division that occurred before lateral root formation (Taylor and Scheuring, 1994).

Analysis of genetic mutants is also an important approach for dissecting the steps in the auxin-mediated lateral root development process (Schiefelbein and Benfey, 1991). A number of mutants that are reduced in their sensitivity to auxin have been identified from several plant species, including tomato (Zobel, 1972) and *Arabidopsis* (Maher and Martindale, 1980; Lincoln et al., 1990; Wilson et al., 1990; Celenza et al., 1995). Examining the expression mode of marker genes for lateral root development in the mutant plants may provide detailed information about the process. In the present study, we further investigated the modes

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of regulation and expression of the *RSI-1* gene.

MATERIALS AND METHODS

Plant Materials and Tissue Cultures

Seeds of the tomato cultivar VFN8 (*Lycopersicon esculentum* Mill cv. VFN8) were obtained from the Petoseed Company (Woodland, CA, USA). The seeds were sterilized by treating them with 50% bleach for 1 h, followed by a brief washing, once with 95% ethanol and four times with sterile deionized water. The seeds were then germinated in liquid MSSV medium (Fillatti et al., 1987). Roots were excised from two-week-old seedlings, and the primary rootstock was maintained as described by Lee et al. (1995).

Arabidopsis thaliana (Lansberg ecotype) seeds were obtained from Dr. Howard Goodman (Harvard Medical School and Massachusetts General Hospital, Boston, USA). The seeds were surface-sterilized by im-mersing them in a solution containing 5% NaOCl and 0.02% Triton X-100 for 10 min, then rinsing them 5 times with sterile water. Sterilized *Arabidopsis* seeds imbibed at 4°C for three days and then grown at 22°C for four weeks in MS liquid medium containing 20% sucrose, under conditions described by Pih et al. (1999).

Northern Blot Analysis

Total RNA was extracted from two-week-old tomato roots using Tri Reagent (Molecular Research Center Inc., Cincinnati, USA), following the manufacturers instruction. For the northern blot analysis, each RNA sample (30 μg of total RNA) was dissolved in 1× MOPS buffer (Taylor et al., 1993). The RNA samples were electrophoresed on a 1.5% agarose-MOPS gel that contained 5.7 M formaldehyde. Uniform loading and integrity of the RNAs were confirmed by examining the intensity of ethidium bromide-bound ribosomal RNA bands under UV light. RNAs on the gel were transferred to a Biotrans polysulfone membrane (Gelman Science, Ann Arbor, USA). The membrane was hybridized to a radiolabeled *RSI-1* cDNA probe (Taylor and Scheuring, 1994), in a solution containing 50% formamide, 0.25 M sodium phosphate buffer (pH 7.2), 0.25 M NaCl, 7% SDS, 20 μg mL^{-1} salmon sperm DNA, and 1 mM EDTA, at 42°C overnight. The blot was washed at 50°C in a solution of 6× SSC and 0.1% SDS for 20 min, 2× SSC and 0.1% SDS for 20 min, and 1× SSC and 0.1% SDS for 20 min. The blot was then exposed to an X-ray film at –70°C for 7 days.

Transformation of the *RSI-1* Promoter-*GUS* Construct into Tomato and *A. thaliana*

A transcriptional fusion of the *RSI-1* promoter-*GUS* reporter gene was constructed and transformed into tomato, as described by Taylor and Scheuring (1994). To transform the fusion construct into *Arabidopsis*, an overnight culture of *Agrobacterium tumefaciens* LBA4404 carrying the *RSI-GUS* binary vector was diluted (1:20) and used to inoculate four-week-old plants grown in liquid MS medium. The roots were cut into 0.5-cm segments and incubated (see Huang and Yeoman, 1984) to induce shoot and root growth. Rooted seedlings were transferred to Magenta GA-7 boxes filled with 50 mL of agar medium that contained 50 μg mL^{-1} kanamycin but no hormone.

To verify the copy number of the *RSI-GUS* fusion gene present in each of the tomato and *Arabidopsis* T1 progeny lines, genomic DNA was isolated and used in Southern blot analyses. The DNA probes were designed from the *RSI-1* promoter or 5′ flanking sequence driving the *GUS* reporter. Copy numbers were determined by comparing the intensity of the probed bands on the blots. In addition, segregation of the GUS activity in the next generation (T2) was analyzed to confirm the copy number in each of the T1 progeny. T1 progenies that showed an approximate 3:1 ratio for GUS expression in the T2 generation were selected for further experiments.

GUS Activity Assays

For in-situ GUS assays, tissues were stained for β-glucuronidase activity (Jefferson, 1987; Kim and Choi, 1998). Photographs were taken with an Olympus SZH-10 stereomicroscope. For the fluorometric GUS assays, tissues were treated, as describe by Jefferson et al. (1987), to determine the rate of 4-methyl-umbelliferone-glucuronide (4-MUG) breakdown by β-glucuronidase activity. Values were taken at 0, 10, 20, and 30 min after addition of the 4-MUG.

RESULTS

Expression Specificity of the *RSI-1* Gene in Wild-Type Tomato Seedlings

The responsiveness of *RSI-1* gene expression to plant hormones was assessed by determining the relative abundance of *RSI-1* mRNA in tomato roots following treatment with various phytohormones for 48

h. These hormones included 5 μM synthetic auxin (naphthaleneacetic acid; NAA), 5 μM 6-benzylaminopurine (BA), 50 μM ABA, 10 μM gibberellic acid (GA), 10 μM ACC (the ethylene precursor), 1 mM salicylic acid (SA), and 10 μM 2,3,5-triiodo benzoic acid (TIBA). A high level of accumulation of *RSI-1* mRNA was observed only with the NAA treatment (Fig. 1A), which was reproducible in other northern assays.

Expression of *RSI-1* in other tissues was examined. Two-week-old seedlings grown in sterile culture were incubated for 48 h in liquid media with or without 5 μM NAA. The seedlings were dissected into roots, hypocotyls, cotyledons, and epicotyls. Total RNA was isolated from each tissue type and hybridized with an *RSI-1* cDNA probe (Fig. 1B). Relatively low levels of *RSI-1* gene expression were observed in all organs in seedlings grown in the absence of NAA. The *RSI-1* gene was induced by NAA, with the highest expression in roots and hypocotyls. About a 14-fold induction in roots and 5- to 6-fold in hypocotyls were observed. The high level of induction in roots and hypocotyls was observed repeatedly in other northern assays, while that in cotyledons was variable and not reproducible.

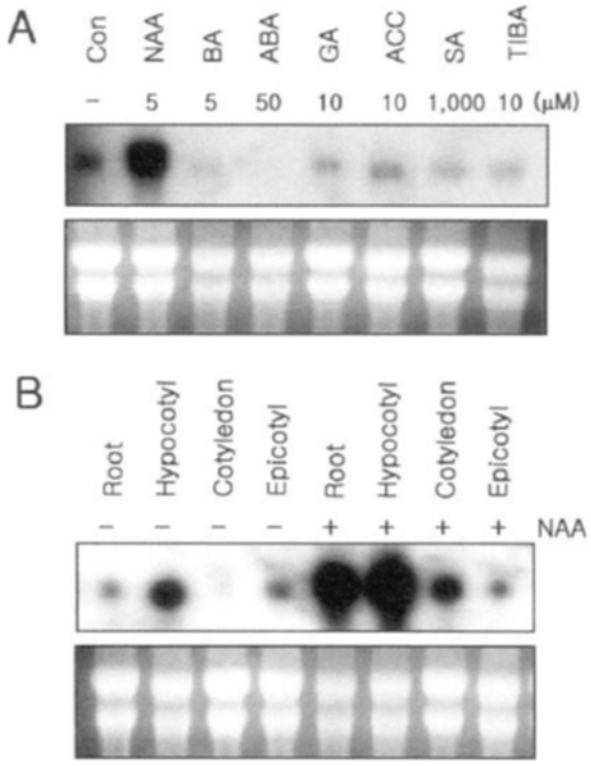

Figure 1. Northern-blot analyses of *RSI-1* gene expression in VFN8 tomato seedlings. The blot with 30 μg/lane of total RNA was probed with the *RSI-1* cDNA. **A.** Effect of various phytohormones on *RSI-1* gene expression in two-week-old roots, treated without or with 5 μM NAA, 5 μM BA, 50 μM ABA, 10 μM GA, 10 μM ACC, 1 mM SA, and 10 μM TIBA for 48 h. **B.** Expression of the *RSI-1* gene in the root, hypocotyl, cotyledon, and epicotyl. Each tissue type was excised from two-week-old seedlings. Ethidium bromide-stained RNA gels are shown as controls for sample loading.

Effect of Auxin Concentration on *RSI-GUS* Expression in Transgenic Tomato Roots

To assess the effect of auxin concentration on *RSI-GUS* activity, roots from the transgenic tomato line, homozygous for one copy of *RSI-GUS*, were treated for 48 h with 0, 1.6, 5.0, or 10.0 μM NAA in liquid culture. A quantitative assessment of GUS expression was obtained with a fluorometric GUS assay (Fig. 2A) using wild-type VFN8 roots as the negative control. The highest induction (3- to 4-fold) of GUS activity was obtained with 5 μM NAA. This was consistent with previous observations that maximum lateral root induction was obtained at the same concentration (Taylor and El-kheir, 1993). In-situ GUS assays were also performed with the transgenic tomato roots to determine detailed GUS expression pattern in the presence of the same concentrations of NAA (Fig. 2B). Without exogenously applied auxin, GUS activity was detected only at the sites of initiation for lateral roots from primary roots, and for adventitious roots from the hypocotyl. With the auxin treatments, high levels of GUS expression were detected in the lateral root and in some portions of the vascular tissue in the primary roots. Exposing the transgenic roots to 1.6 μM or 5 μM NAA for 48 h increased the frequency of lateral root initials, with a corresponding increase in the intensity of GUS activity.

Expression of the *RSI-GUS* Gene in Transgenic *Arabidopsis*

The transcriptional fusion of the *RSI-1* promoter-*GUS* reporter gene was transformed into four-week-old *A. thaliana*. In the absence of any hormone treatment, GUS activity was observed at the site of lateral root initiations in the vascular tissue of roots and hypocotyls (Fig. 3A). GUS activity was strongly enhanced in the presence of 5 μM NAA (Fig. 3B). An increased number of primordia exhibited high levels of GUS activity in lateral roots (Fig. 3C) and in adventitious roots from the hypocotyls (Fig. 3D).

Database Search for *RSI-1* Homologues in *Arabidopsis*

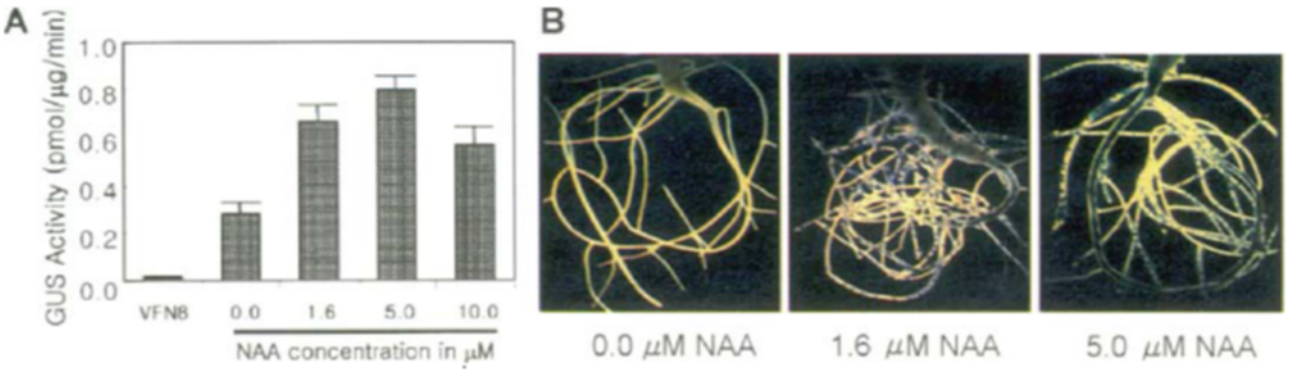

Figure 2. Effect of auxin concentration on *RSI-GUS* expression in two-week-old tomato seedlings. **A.** Fluorometric GUS assay. Each bar represents the mean of six replicates and the standard deviation. **B.** In-situ RSI-GUS localization in roots. Seedling roots were incubated with 0, 1.6, or 5.0 μM of NAA for 48 h, then stained for GUS activity.

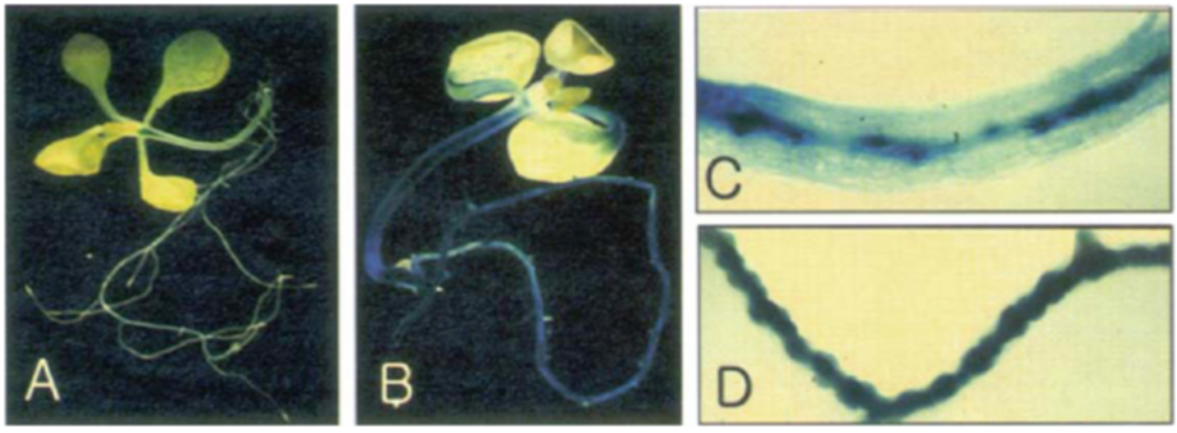

Figure 3. In-situ localization of the RSI-GUS activity in a transgenic *Arabidopsis* seedling grown in the absence (A) or presence (B) of 5 μM NAA for 48 h. A root (C) and a hypocotyl (D) segment of the *Arabidopsis* seedling treated with 5 μM NAA for 48 h.

A database search using the BLASTX program revealed several plant genes highly homologous to the tomato *RSI-1* gene (Table 1). In amino acid sequences, RSI-1 protein exhibits high homology with the *Arabidopsis* GASA5 protein and its homologues identified from *Picea mariana*. In addition to GASA5,

Table 1. Amino acid sequences producing significant alignments with tomato RSI-1 (Genbank accession number L22189).

Acc. No.[a]	Description	Organism	Score (bits)	Identity[b]
X63093	GAST1	Tomato	125	48/60 (80)
AF051227	GASA5-like	*Picea mariana*	125	49/63 (77)
X77225	GIP1	petunia	124	48/59 (81)
U53221	GASA5	*Arabidopsis*	121	49/68 (72)
AC004669	unknown	*Arabidopsis*	111	41/63 (65)
X98255	stimulated by GA	*Arabidopsis*	94	45/98 (45)
U15683	GASA4	*Arabidopsis*	94	37/63 (58)
AC004218	GASA5-like	*Arabidopsis*	65	22/56 (39)
U11765	GASA2	*Arabidopsis*	64	30/68 (44)
U11764	GASA3	*Arabidopsis*	62	30/68 (44)
AC005396	GAST1-like	*Arabidopsis*	60	22/54 (40)
U11766	GASA1	*Arabidopsis*	55	25/63 (39)

[a]Genbank accession number.
[b]Number of identical amino acid residues/Total number of residues in DNA fragment analyzed (%).

other proteins are encoded by the *Arabidopsis* GASA gene family (Herzog et al., 1995), including GASA1, GASA2, GASA3, and GASA4. These also showed homology with RSI-1. Tomato GAST1 (Shi et al., 1992) and petunia GIP (Ben-Nissan and Weiss, 1996) also showed strong homology with RSI-1 in their deduced amino acid sequences. The RSI-1 protein exhibited overall 79% amino acid identity to the GAST1 protein.

DISCUSSION

The *RSI-1* gene was induced in roots specifically by auxin, but not by other phytohormones, such as GA, ABA, BA, SA, cytokinin, the ethylene precursor ACC, and the auxin polar transport inhibitor TIBA (Fig. 1A). In tomato seedlings, exogenously applied auxin enhanced transcription of the *RSI-1* gene in roots and hypocotyls, but not in cotyledons and epicotyls (Fig. 1B). The accumulation of *RSI-1* transcripts started within 4 h of the auxin treatment, and preceded noticeable formation of new lateral roots (data not shown). To date, the *RSI-1* gene is one of the earliest genes expressed in the process of lateral root formation and, thus, is a good marker for lateral root initiation.

In transgenic tomato plants, the expression patterns of the *GUS* gene fused to the *RSI-1* promoter included dependence on auxin concentration (Fig. 2A), tissue specificity (Fig. 2B), and expression timing in tissue development (data not shown). These patterns were consistent with the results obtained from the northern blot analyses (Fig. 1). Thus, the *RSI-GUS* expression pattern observed in transgenic plants reflects the actual regulation of the endogenous *RSI-1* gene. Moreover, transgenic roots grown in the absence of auxin also showed GUS expression at the sites of lateral root initiation (Fig. 2A). This suggests that the molecular mechanisms involved in lateral root initiation, induced by either endogenous or exogenously applied auxin, are similar.

A database search revealed several plant genes that were highly homologous to the tomato *RSI-1* gene (Table 1). The *GASA* genes are structurally related to the *GAST1* gene from tomato (Shi et al., 1992). Although function of the protein is unknown, the *GAST1* gene reportedly is induced by gibberellic acid. (Shi et al., 1992). Another gene homologous to the *RSI-1* gene, identified from petunia (*GIP*), is also GA-inducible (Ben-Nissan and Weiss, 1996). Tissue specificity differs remarkably between the auxin-regulated *RSI-1* gene and the GA-regulated *GASA* gene family. As described, the *RSI-1* gene is highly expressed in roots and hypocotyls, but not in cotyledons and epicotyls. In contrast, *Arabidopsis GASA1* transcripts greatly accumulated in siliques, whereas *GASA4* transcripts were detected mainly in flower buds, and *GASA3* transcripts accumulated at the end of the maturation stage of the silique (Aubert et al., 1998). In tomato, the *GAST1* gene is not expressed at a detectable level in roots (Shi et al., 1992).

Results from studies with auxin suggest that auxin-activated signal transduction pathways and regulation mechanisms for gene expression may differ in each tissue, even for exerting similar physiological responses. For instance, differential expression of two auxin-regulated genes, *SAURs* and *GH3*, has been demonstrated in different soybean organs (Gee et al., 1991).

The same tissue specificity that had been identified in tomato was observed in *Arabidopsis* transformed with the *RSI-GUS* construct (Fig. 2). Exogenously applied auxin enhanced GUS activity in roots and hypocotyls, but not in cotyledons and epicotyls. The overall expression pattern of the *RSI-GUS* fusion gene including inducibility by auxin (Fig. 2), tissue specificity (Fig. 3), and expression timing in tissue development (data not shown), was consistent with the results obtained from study with RSI transgenic tomato plants These results suggest that *cis*- and *trans*-acting regulatory elements responsible for lateral root initiation are conserved in both plants. Therefore, we postulate that gene(s) homologous to the tomato *RSI-1*, other than from the GA-regulated *GASA* gene family, are present in the *Arabidopsis* genome.

Examining the expression mode of marker genes for lateral root development in mutant plants provides interesting, detailed information about the process. The tomato mutant *diageotropica* (*dgt*) has been characterized physiologically and biochemically (Zobel, 1972; Ursin and Bradford, 1989). This mutant plant is defective in some primary events in auxin actions (Kelly and Bradford, 1986; Hicks et al., 1989; Coenen and Lomax, 1998).

Three mutants isolated from *Arabidopsis--aux1* (Maher and Martindale, 1980), *axr1* (Lincoln et al., 1990), and *axr2* (Wilson et al., 1990)--have been well characterized in terms of their root-development phenotypes. Recently, *Arabidopsis* mutants that are defective in lateral root development have been isolated (Celenza et al., 1995). These mutants, *alf1-1*, *alf3-1*, and *alf4-1*, have helped elucidate the developmental processes for lateral roots. The *alf1-1* mutation caused hyperproliferation of lateral roots, *alf3-1* was defective in the maturation of lateral roots, and *alf4-1*

prevented initiation of lateral roots. Study of these mutants revealed that auxin was required for the initiation, morphogenesis, and continued viability of lateral roots. Thus, investigating the expression mode of marker genes, such as the *RSI-1*gene, in these mutant plants will provide detailed information about the lateral root development process.

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