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Expression of the *RSI-1* Gene during Development of Roots and Reproductive Organs in Tomato

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The *RSI-1* gene is expressed in pericycle cells just prior to the first round of cell division for lateral root development in tomato. We transformed tomato plants with the *RSI-1* gene promoter linked to a GUS reporter gene. GUS activity was detected not only at the sites of initiation for lateral and adventitious roots, but also at the primary root tip. Expression of the fusion gene was also regulated at various stages of tissue development: in particular, during the formation of reproductive organs such as pollen and fruit. Overexpression of the *RSI-1* gene in either the sense or antisense orientation resulted in arrest of fruit development and seed germination. The *RSI-1* gene product, therefore, may play a role in auxin-induced cell division in various developing tissues. Inter- and intramolecular disulfide bridges between cysteines rich in the RSI-1 protein might be involved in cell-wall modifications that are essential for new cell division. These hypotheses for the role of the *RSI-1* gene in lateral-root and reproductive-organ development remain to be tested.

Keywords: auxin, lateral root, reproductive organs, tomato, transgenic plant

The *RSI-1* gene has been identified as a molecular marker for lateral root initiation in tomato (Taylor and Scheuring, 1994). Lateral root initiation involves a series of rapid cell divisions in the parent root pericycle near the xylem poles of the vascular cylinder (Charlton, 1991; Laskowski et al., 1995). The *RSI-1* gene was detected in a small subset of pericycle cells just prior to undergoing a first round of cell division that occurs before lateral root formation. Expression of the gene is induced specifically by auxin in the early developmental stages of lateral roots and adventitious roots from hypocotyls (Cheong et al., 1999). However, the role of the RSI-1 protein in the developmental process is not known.

The plant hormone auxin may be a signal for lateral root initiation. Exogenous applications of auxin dramatically increased the frequency of lateral root formation in most dicot plants (Hinchee and Rost, 1986; Blakely et al., 1988; MacIsaac et al., 1989; Taylor and El-Kheir, 1993). Moreover, transgenic plants that overexpressed bacterial auxin biosynthetic genes exhibited increased lateral root formation (Klee et al., 1987; Kares et al., 1990). Based on a study of *Arabidopsis* mutants with defective lateral root development, auxin is thought to be required for initiating and promoting cell division in the pericycle of developing lateral roots (Celenza et al., 1995).

Auxin acts as a signal for cell division, elongation, and differentiation (Walker and Estelle, 1998; Guilfoyle, 1998). It plays a key role in diverse developmental processes, including tissue differentiation, root development, apical dominance, tropisms, senescence, flowering, and fertility (Garbers and Simmons, 1994; Walker and Estelle, 1998).

Diverse signaling pathways are present in plants, which lead to such various auxin responses in different tissues and organs (Macdonald, 1997; Guilfoyle et al., 1998). Differential expression of a number of auxin-regulated genes has been demonstrated in soybean (Gee et al., 1991) and tomato (Mito and Bennett, 1995). Although the *RSI-1* gene was originally identified in the root-initiation process, it should be tested to see whether its expression is limited to root development or if it is involved more generally in auxin-induced cell divisions in developing tissues. Investigation of tissue specificity is one of the important steps in elucidating the exact role of the RSI-1 protein during developmental processes.

Auxin is not the only plant hormone required for cell division. Studies with mutants in the gibberellic acid (GA) synthesis pathway have indicated that some

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GA-regulated genes also play a role in regulating cell division and elongation (Aubert et al., 1998). Some GA-inducible genes, including Tomato *GAST1* (Shi et al., 1992) and those of the *Arabidopsis GASA* family (Herzog et al., 1995), show high homology with the auxin-regulated *RSI-1* gene in their amino acid sequences. The *RSI-1* and *GASA* genes encode proteins with similar activities but which may be independently regulated by the two types of phytohormone in different tissues and at different developmental stages (Cheong et al., 1999). Therefore, tissue specificity for expression of the *RSI-1* gene must be compared with that of other GA-inducible homologues during tissue development.

In the present study, we further investigated the modes of regulation and expression of the *RSI-1* gene at various stages of plant development. We also proposed the possibility that the *RSI-1* gene product plays a role in cell-wall modification, thereby allowing new cell division during lateral-root and reproductive-organ development.

MATERIALS AND METHODS

Plant Tissue Culture

Seeds of tomato cultivar VFN8 (*Lycopersicon esculentum* Mill cv. VFN8) were obtained from the Petoseed Company (Woodland, CA, USA). For experiments with cultured tissues, the seeds were sterilized by treating them with 50% bleach for 1 h, followed by a brief wash once with 95% ethanol and four times with sterile deionized water. The seeds were then germinated in liquid MSSV medium (Fillatti et al., 1987).

Transformation of the *RSI-1* Promoter-GUS Construct

A transcriptional fusion of the *RSI-1* promoter-*GUS* reporter gene was constructed and transformed into tomato plants, as described by Taylor and Scheuring (1994). We examined the copy number of the *RSI-GUS* fusion gene present in each of the T1 progeny lines by comparing the intensity of the probed bands on Southern blots and by observing the segregation of GUS activity in the next generation. Two individual transgenic lines, homozygous for one copy of the *RSI-GUS* insertion (including *RSI-GUS* 16A-6), and 2 other individual transgenic lines that showed multiple (4 to 5) copies were isolated. The *RSI-GUS* 16A-6 line was used in all of our experiments.

GUS Activity Assays

For in-situ assays, tissues were stained for β-glucuronidase activity according to Dietrich et al. (1992). Cytological analysis of GUS gene expression was performed as described by Lee et al. (1995). Photographs were taken with an Olympus SZH-10 stereomicroscope.

Transformation of *RSI-1* Sense and Antisense Constructs

A BamHI-SstI fragment containing the *RSI-1* genomic clone (Taylor and Scheuring, 1994) was inserted into the pBI220.7 vector (Bevan, 1984) at the corresponding restriction enzyme sites to create a sense construct. The pBI220.7 vector contained the proximal regions and three repeats of the enhancer sequences of the CaMV promoter. The antisense construct consisted of the same region, in the reverse orientation, inserted into the pBI220.7 vector with BamHI-KpnI ends. Both sense and antisense constructs were inserted into BIN19 as HindIII/EcoRI fragments, then electroporated into *Agrobacterium tumefaciens* LBA4404, as described by Mersereau et al. (1990). Transformation of *Agrobacterium* into the tomato plants and regeneration of these plants followed the procedures of Taylor and Scheuring (1994).

RESULTS

Transformation of the *RSI-1* Promoter-*GUS* Construct

RSI-1 is present as a single-copy gene in the tomato plant genome (Taylor and Scheuring, 1994). We assumed that a line homozygous for one copy of *RSI-GUS* would express the reporter with a pattern and intensity similar to the original *RSI-1* gene during plant development. All progeny from the homozygous line were expected to behave similarly and to provide reliable results for further GUS assays. The degree of GUS activity varied between transformants that contained different copy numbers of *RSI-GUS*. Auxin inducibility and tissue specificity of the gene expression, however, remained constant, as was previously observed by Taylor and Scheuring (1994). This indicates that expression was largely promoter-driven rather than due to the position of the

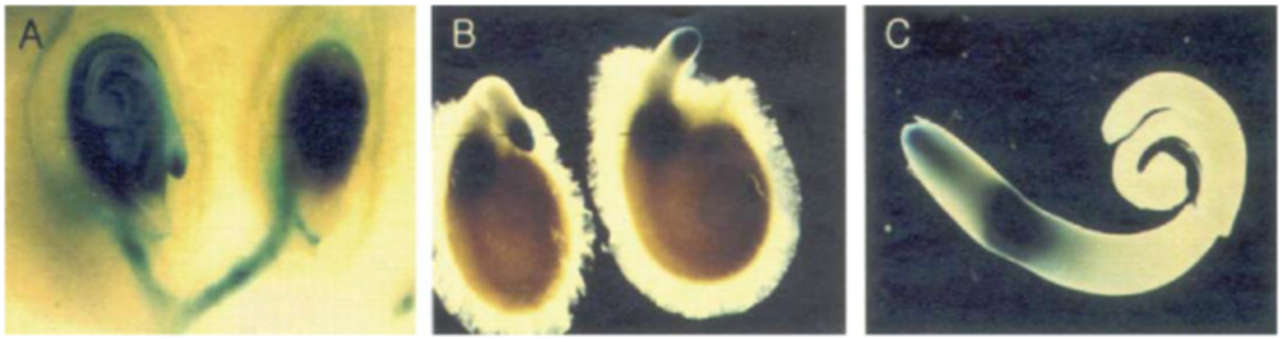

Figure 1. In-situ localization of *RSI-GUS* activity in a developing embryo from a transgenic tomato. **A.** Embryo in late stage of development. **B.** Two-day-old seedling after germination. **C.** Two-day-old seedling with seed coat and endosperm removed.

insertion site.

GUS activity expressed at various developmental stages was analyzed in four transgenic tomato plants known to have different copy numbers of the *RSI-GUS* transgene. The spatial distribution of GUS activity in 50 progeny (T1 generation) from each of the transgenic plants was determined histochemically by in-situ GUS assay. The level of GUS activity was proportional to the copy number of the *RSI-GUS* gene, but the general expression patterns were the same.

Expression of the *RSI-GUS* Gene in Developing Embryos and Roots

In developing embryos from the *RSI-GUS* 16A-6 line, GUS activity was detected at the tips of radicles and at the junction regions between radicle and hypocotyl (Fig. 1A). This pattern continued after germination (Fig. 1, B and C). In the two-week-old seedlings, high levels of GUS activity were detected at sites of lateral root initiation (Fig. 2, A and B), and at columellar regions of root caps and meristems in root tips (Fig. 2C). In addition, transverse sections through hypocotyls revealed that adventitious roots were initiated near the xylem bundles (Fig. 2D). The hypocotyls had four xylem bundles; two adventitious root primordia grew near two of these xylem bundles. Strong GUS expression was detected in that region. Relatively weak but significant GUS expression near the other two xylem bundles indicated that this region also was undergoing adventitious root initiation.

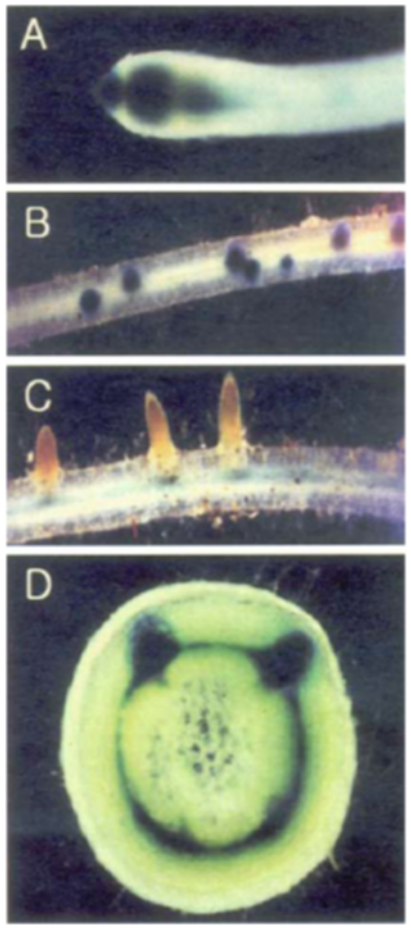

Figure 2. In-situ localization of *RSI-GUS* activity in the developing root and hypocotyl of a two-week-old transgenic tomato seedling. **A.** Root in early stage of lateral root initiation. **B.** Root with emerging lateral roots. **C.** Primary root tip. **D.** Transverse section through hypocotyl.

Expression of the *RSI-GUS* Gene in Developing Flowers

Flowers from the adult transgenic plant *RSI-GUS* 16A-6 were analyzed for GUS activity at different stages of development. At the first stage, petals were green and not fully developed. In the second stage, petals were fully developed in size but still closed, and were greenish-white (Fig. 3A). In the third stage,

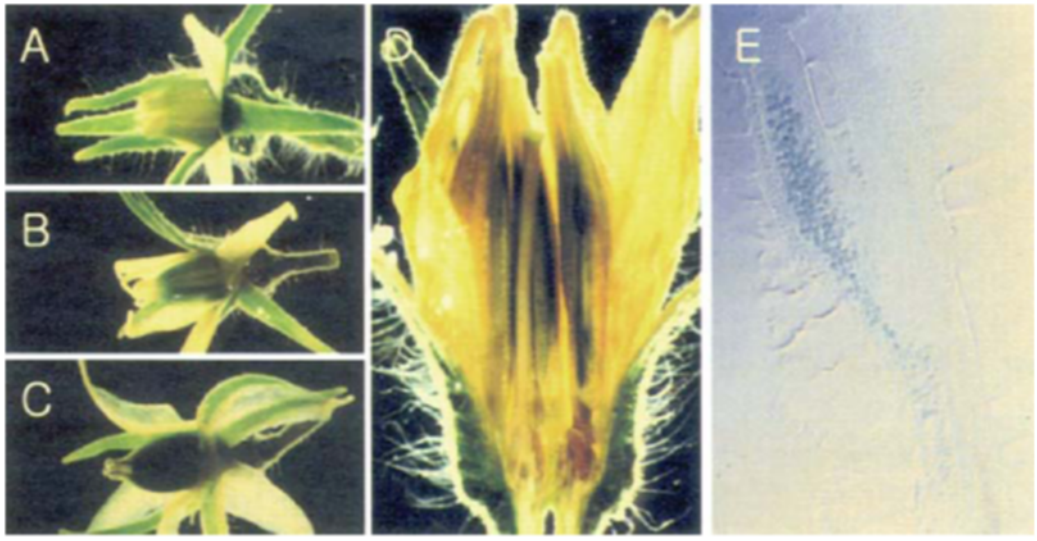

Figure 3. *RSI-GUS* activity in developing tomato flowers. **A.** Flower in early stage. **B.** Flower in fully blooming stage. **C.** Withering flower. **D.** Longitudinal section through a flower in fully blooming stage. **E.** Longitudinal section through a tomato anther sac (cytological analysis).

petals were yellow and the flower was open (Fig. 3B). In the fourth stage, petals were withering and the tips of anthers were starting to dry (Fig. 3C).

A low level of GUS activity was detected in the anthers from the second stage onward, with strongest activity in the fourth stage. A cross section through a third-stage flower showed that GUS activity was detected only in the anther sac, but not in other floral parts (Fig. 3D). At this stage, strong blue staining in the anthers corresponded to pollen grains; weak staining was observed in the tapetum (Fig. 3E). Pollen from a mature anther showed a population segregating for presence or absence of GUS activity (data not shown).

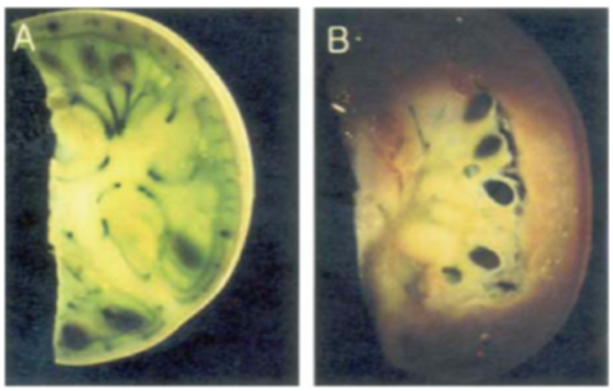

Figure 4. In-situ localization of *RSI-GUS* activity in developing tomato fruits. **A.** Section through an immature green fruit. **B.** Section through a mature red fruit.

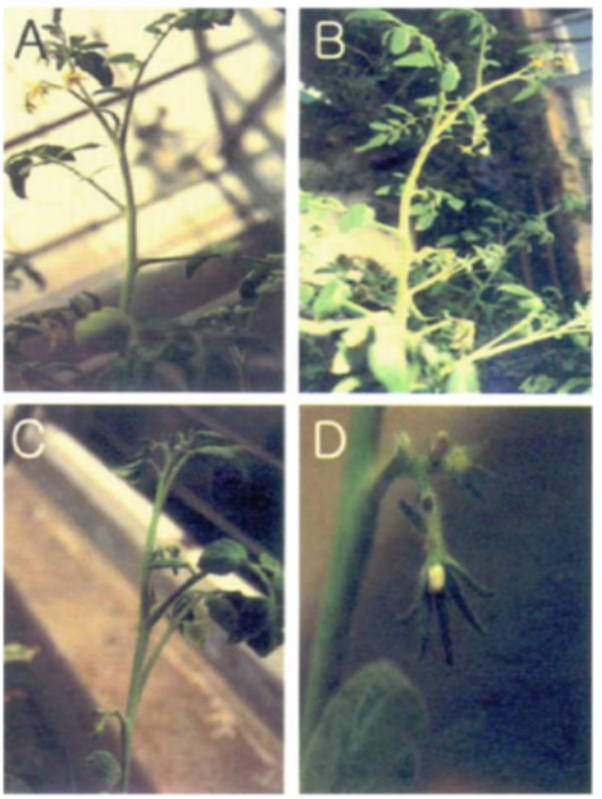

Figure 5. Alterations of fruit development by overexpression of sense or antisense *RSI-1* cDNA constructs in transgenic tomato plants. **A.** Wild-type tomato plant showing flowering branch and large-sized green fruit. **B.** Antisense transgenic plant with four flowering branches. **C.** Sense transgenic plant with three flowering branches. **D.** Arrested fruit development in sense transgenic plants.

Expression of the *RSI-GUS* Gene in Fruit Development

During fruit development, GUS expression was detected in vascular bundles that connected developing seeds to the placenta (Fig. 4A). In ripe fruit, GUS activity was absent from the vascular bundles, but was still present where the seed was connected to vascular tissue of the fruit (Fig. 4B).

Overexpression of Antisense or Sense *RSI-1* cDNA Constructs

To understand the role of the *RSI-1* gene during plant development, sense or antisense construct of the *RSI-1* gene was overexpressed in transgenic tomato plants. Two individual transformants for each antisense and sense construct were examined. Southern analysis showed that these original transformants contained one or two copies of the insert (data not shown). Mature plants from either construct type exhibited no significant morphological changes in vegetative phenotypes, compared with wild-type plants. However, notable changes were detected in developing flowers.

Typically, in wild-type tomato plantss, the second-latest flowering branch underwent fruit development while the latest flowering branch was blooming (Fig. 5A) and a small, growing ovary was detected in the second-latest flowering branch. However, in the transgenic plants, this pattern was not observed. In both sense and antisense transgenic plants, growing fruit was not noticeable, even in the fifth-latest flowering branch (Fig. 5, B and C). Most ovaries had arrested development (Fig. 5D) or were senescent. A few ovaries grew larger, but rotted because the whole growing process was slower than normal (not shown). A single fruit that reached maturity contained a small number of seeds, but the seeds failed to germinate. Overall, mature transformants of either sense or antisense plants exhibited almost identical phenotypes in flower and fruit development.

DISCUSSION

RSI-GUS expression was first observed in pericycle cells that initiated lateral root primordia in transgenic tomatoes (Taylor and Scheuring, 1994). In the present study, GUS activity was detected not only at the sites of lateral root initiation (Fig. 2, A and B), but also at primary root tips (Figs. 1, and 2C) and at the sites of adventitious root initiation in hypocotyls (Fig. 2D). This sug- gests that expression of the *RSI-1* gene is not restricted to lateral roots but, instead, the gene might be involved in general root-initiation processes.

RSI-GUS activity was detected in pericycle cells before the first cell division (Taylor and Scheuring, 1994). Additionally, in *Arabidopsis* root mutants, auxin was required in lateral root development to initiate cell division in the pericycle (Celenza et al., 1995). Recently, it was observed that *RSI-1* is induced specifically by auxin (Cheong et al., 1999). Thus, the *RSI-1* gene product might play a role in auxin-induced cell division processes in developing tissues, including lateral roots.

We examined whether the *RSI-1* gene in tomato is expressed in other tissues undergoing active cell division. GUS activity was detected in the late stages of pollen-grain development (Fig. 3E), and in the vascular tissues of immature green fruit (Fig. 4A). GUS activity disappeared as the fruit matured (Fig. 4B). Overexpressing the *RSI-1* gene, in either sense or antisense orientation, resulted in notable changes in developing flowers and fruits (Fig. 5). Therefore, the *RSI-1* gene product is probably involved generally in cell division throughout developing tissues.

Several genes have been identified as having high homology with *RSI-1* in their amino acid sequences. These genes, which are induced by GA, include those of the *Arabidopsis GASA* gene family (Herzog et al., 1995), *GASA* homologues in *Picea mariana* and tomato *GAST1* (Shi et al., 1992), and petunia GIP (Ben-Nissan and Weiss, 1996). Among these, *GASA4* gene expression has been detected in all meristematic regions, including vegetative, inflorescence and floral meristems, as well as primary and lateral root tips (Aubert et al., 1998). The GASA4 protein probably also plays a role in cell division, rather than simply in elongation (Aubert et al., 1998). Therefore, it is possible that auxin-regulated *RSI-1* and GA-regulated *GASA4* encode proteins that have similar activities during cell division, but are independently regulated by the two types of phytohormone.

During lateral root formation, the cell-wall structure of pericycle cells must be altered to allow new cell division. This was demonstrated in carrot cells, which in suspension culture divided to form a somatic embryo (Cordewener et al., 1991; van Engelen and de Vries, 1992). Similar changes may occur during lateral-root initiation and reproductive-organ formation. For example, bean extensins are expressed at the sites of lateral root development (Keller and Lamb, 1989). Solanaceous lectins are also high in cys-

teine, and the cell wall proline-rich proteins (PRPs) and extensins are high in basic amino acids (Showalter, 1993).

The RSI-1 protein is also cysteine-rich and highly basic (Taylor and Scheuring, 1994). Inter- and intramolecular disulfide bridges between cysteines in the RSI-1 protein might be involved in cell-wall modification. In the present study, mature transformants of either sense or antisense plants exhibited identical phenotypes in flower and fruit development (Fig. 5). Based on this, we hypothesize that the *RSI-1* gene might be involved, during cell division, as a cell-wall structural protein rather than as a regulatory protein. This hypothesis remains to be tested. In this regard, subcellular localization of the RSI-1 protein may provide more detailed information of the function of *RSI-1*.

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