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Analysis of the Tissue-Specific *S* RNase Gene Promoter Associated with Self-Incompatibility in Tomato (*Lycopersicon peruvianum* L.)

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To understand the expression pattern of the *S* RNase gene in the floral tissues associated with self-incompatibility (SI), promoter region of S_{11} RNase gene was serially deleted and fused GUS. Five chimeric constructs containing a deleted promoter region of the S_{11} RNase gene were constructed, and introduced into *Nicotiana tabacum* using Agrobacterium-mediated transformation. Northern blot analysis revealed that the GUS gene was expressed in the style, anther, and developing pollen of all stages in each transgenic tobacco plant. The developing pollen expressed the same amount of GUS mRNA in all stages in transgenic tobacco plants. In addition, histochemical analysis showed GUS gene expression in vascular bundle, endothecium, stomium, and tapetum cells during pollen development in transgenic plants. From these results, it is speculated that SI of *Lycopersicon peruvianum* may occur through the interaction of *S* RNase expressed in both style and pollen tissues.

Keywords: *Lycopersicon peruvianum*, Self-Incompatibility (SI), *S* RNase gene promoter

Many flowering plants possess genetically controlled self-incompatibility (SI) systems that prevent inbreeding. SI is often controlled by a single gene locus known as the *S* locus (Haring et al., 1990). SI systems are classified as either gametophytic or sporophytic based on the ability of the style to discern the presence of self-pollen and on the female tissue's capacity to inhibit the growth or germination of self-pollen. In gametophytically determined systems such as Solanaceous plants, pollen expresses its own haploid genotype, and mating is incompatible if the *S* allele of the pollen is matched by one of the two alleles in the diploid tissue of the style (De Nettancourt, 1977; Chung and Shin, 1997).

Lycopersicon peruvianum has a gametophytic SI system genetically controlled by a single locus with multiple alleles (*S* alleles) (Tanksley and Loaiza-Figueroa, 1985). Three cDNAs and two genomic genes coding for *S* glycoproteins of *L. peruvianum* have previously been isolated and have shown that they cosegregate with their respective *S* alleles in genetic crossing (Chung et al., 1994, 1995). Recently, the inheritance of SI was studied by crossing two accessions of *L. peruvianum* (Kowyama et al., 1994). One accession was, like most *L. peruvianum* accessions, self-incompatible, and the other was self-compatible. Self-compatibility (SC) segregated in these crosses as a non-functional allele of the *S* locus, and it was also found that the *S* RNase encoded by this allele was enzymatically inactive, presumably due to a change in one of the catalytically important amino acids. The supportive result was reported by Huang et al. (1994) in which an inactive form of Petunia *S* RNase was introduced into the transgenic *Petunia inflata plants*, and the loss of RNase activity was correlated with an inability to reject pollen bearing the matching *S* allele.

One characteristic feature of SI is the precise pattern of temporal and tissue-specific expression of the *S*-locus. In previous work (Chung et al., 1995), we found the highly homologous regions in −360 to −310, −215 to −189, and −151 to −123 bp from the ATG codon between two *S* RNase gene promoters, suggesting that these sequences might control the expression of the genes for *S* RNase in the style tissue of *L. peruvianum*.

To further understand the patterns of tissue specific expression on the promoter sequence of the *S* RNase gene, a promoter region of the S_{11} RNase gene was deleted on the basis of highly homologous promoter sequences between the S_{11} and S_{12} RNase genes. Five deletion fragments from promoter region of the S_{11} RNase gene were constructed with a plant expression vector by PCR, and introduced into the leaf discs of *Nicotiana tabacum* using *Agrobacterium*. GUS gene expression in the style and developing pollen during all developmental stages in each transgenic tobacco

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plant was demonstrated clearly by GUS histochemical assay and Northern blot analysis.

MATERIALS AND METHODS

Plant Material

Transgenic tobacco plants (*N. tabacum* cv. Xanthi) introduced with five chimeric constructs were grown in greenhouse. At the flowering stage, styles, anthers, pollens, and leaf tissues of each transgenic plant were collected and used as experimental materials.

Plant Transformation

The recombinant Ti-plasmid pBI 101 with five chimeric constructs was transferred into *Agrobacterium tumefaciens* LBA 4404 by the triparental mating method using a helper plasmid pRK 2013 (Fraley et al., 1983). Leaf discs of *N. tabacum* were infected with the *Agrobacterium* co-cultivation method (Horsch et al., 1985). The infected leaf discs were grown on a Murashige and Skoog (MS) medium supplemented with benzylaminopurine (1.0 mg/L) and naphthalene acetic acid (75 mg/L). Shoots were regenerated on a fresh MS medium supplemented with kanamycin (400 mg/L) and carbenicillin (500 mg/L). Regenerated shoots were regenerated on a fresh MS medium containing the same concentrations of antibiotics to induce root formation. The kanamycin resistant plants and untransformed plants were transferred separately to soil after rooting was maintained in the green house.

PCR Analysis

Total leaf DNA (1 μg) isolated from each transgenic plant was amplified with two unique GUS gene regions by PCR. The sequences of GUS gene primers used in this study were: Forward: 5'-CAGTCTG-GATCGCGAAA-3' and Reverse: 5'-GCTTCGAAAC-CAATGCC-3'.

Reaction conditions, buffers, and sample preparation for PCR were as suggested by the manufacturer of Taq polymerase (Perkin Elmer). All primers and dNTPs were diluted from stock concentration using sterile distilled water. The final reaction volume was 50 μL, including 2 μL of sample DNA (1 μg/μL). The amplification was performed for 30 cycles with each cycle comprising a 1 min denaturation step at 94°C, followed by a 1.5 min annealing step at 65°C and a 2.5 min primer extension step at 72°C. Reaction was done using a DNA Thermal Cycler 2400 (Perkin Elmer). The PCR products were separated by electrophoresis in a 1.5% (W/V) agarose gel and visualized with ethidium bromide on an UV illuminator.

Northern Blot Analysis

Total RNA (5 μg) was isolated from stigma, anthers of five developmental stages and mature pollen in each transgenic plant. Poly $(A)^+$ RNAs were electrophoresed on a 1% formaldehyde gel and transferred into a N^+ nylon membrane (Amersham). For hybridization, the GUS region in pBI 121 digested with restriction enzymes HindIII and SacI was used as probes. Prehybridization was performed at 65°C for 4 h followed by hybridization at the same temperature for 16 h with random-primed ^{32}P-labeled primers. Membranes were washed twice in 2× SSC, 0.1% SDS and 1× SSC, 0.1% SDS, respectively, and autoradiographed for three days with an intensifying screen at –80°C.

Histochemical Assay of GUS Activity

Anther, pollen, and stigma tissues in each transgenic plant with deleted S_{11} RNase promoters/GUS constructs were soaked in the histochemical reagent X-Gluc (5-bromo 4-chloro 3-indolyglucuronide) and incubated at 37°C for 1 day in the dark. Tissues were bleached by soaking in ethanol (70%) for five times at 70°C (Gallagher, 1992; Jeon et al.,1994), embedded with paraffin, and sectioned by a Microtome. The sectioned samples were photographed with a microscope (Zeiss 2000).

RESULTS

Construction of Deletion Fragments and Transformation of *N. tabacum*

Two genomic DNA fragments of 2.1 and 1.3 kb containing the promoter regions of S_{11} and S_{12} RNase genes were previously sequenced and characterized (Chung et al., 1995). Two promoter regions shared homologous sequences around regions 300 bp upstream of ATG, which might control the tissue-specific expression of the *S* locus. For further investigation of these unique sequences, five deletion fragments containing homologous sequences between the S_{11} and S_{12} promoter regions were amplified from

Figure 1. PCR analysis of each transgenic plant with two unique GUS primers. PCR products showed 1.94 kb in size of GUS gene. Lane N indicates the total genomic DNA of the non-transformed tobacco plant. Lane P was loaded the PCR product from plasmid DNA of PBI 101. Lane P1 was loaded the PCR product from transgenic tobacco plant DNA with pBI 101 construct. Lanes 1-6, transgenic tobacco plants with pSP 1 (728 bp); lanes 7-12, transgenic tobacco plants with pSP 2 (396 bp); lanes 13-15, transgenic tobacco plants with pSP 3 (253 bp); lanes 16-23, transgenic tobacco plants with pSP 4 (188 bp); lanes 24-27, transgenic tobacco plants with pSP 5 (127 bp).

the S_{11} RNase gene by PCR. For cloning into the plant transformation vector (pBI101), two unique restriction enzyme sites, HindIII and SalI, were added in the region of 5' and 3' ends in PCR primers. As a result, five deletion mutants containing different lengths of the S_{11} RNase gene promoter fused with the GUS gene were generated. These five chimeric constructs were designated as pSP 1 to 5, and were used as donor for *Agrobacterium* mediated transformation. Deletion fragments 396 (pSP 2), 253 (pSP 3), and 188 (pSP 4) bp in length were designed according to the homologous sequences in the 5' upstream region of the S_{11} and S_{12} genes. Fragments 728 (pSP 1) and 127 (pSP 5) bp were randomly chosen in promoter region of S_{11} genomic gene. The Ti-plasmids containing five chimeric constructs were introduced into *N. tabacum* plants via *Agrobacterium*-mediated transformation, and 2 or 3 tansgenic plants that showed GUS activity was obtained. The presence of the GUS gene sequence incorporated into the transgenic plants was confirmed by PCR using specific oligonucleotide probes of the GUS gene (Fig. 1).

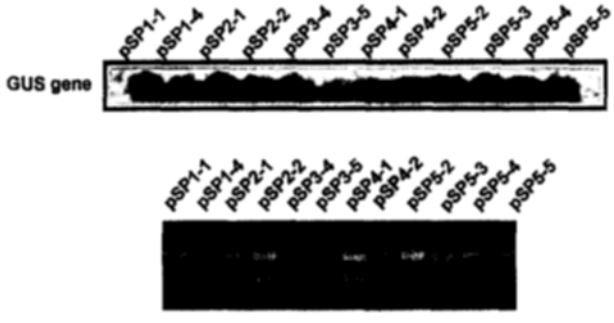

Figure 2. Northern blot analysis of chimeric S_{11} promoter/GUS fusion genes with a GUS gene probe in the style of each transgenic plant. pSP 1-5 indicate in the number of transgenic plants transformed with each S_{11} promoter region. pSP 1-1 and 1-4 indicated in the 728 bp of S_{11} promoter region. pSP 2-1 and 2-2, 396 bp; pSP 3-4 and 3-5, 253 bp; pSP 4-1 and 4-2, 188 bp; pSP 5-3, 5-4 and 5-5, 127 bp.

Expression of the Introduced Five Chimeric Constructs in Styles of Transgenic Tobacco

The stylar expression of the chimeric constructs was characterized with 12 transgenic plants that appeared to have GUS activity. All 12 transgenic plants had transcript in stylar tissue as expected. The expression level of GUS gene in each transgenic plant was similar, indicating that only the 127 bp upstream region from ATG of *S* RNase gene promoter is enough to control the tissue-specific expression in style tissue (Fig. 2).

Tissue-Specific Expression in Pollen of Transgenic Tobacco

In previous works in which genomic S_{11} RNase gene was introduced into *N. tabacum*, it was shown to be regulated developmentally in the stylar tissue (Chung et al., 1993, 1994). The tissue specificity of S_{11} RNase gene expression in transgenic plants was similar to that in *L. peruvianum*, except that the S_{11} RNase mRNA was not previously detected in pollen of *L. peruvianum* (Chung et al., 1993, 1994).

To further examine the precise expression pattern in pollen, five chimeric constructs were used. Northern blot analysis with five deleted S_{11} RNase gene promoter/GUS fusion genes revealed that the GUS gene was expressed in all stages of pollen development in each transgenic plant (Fig. 3), while no

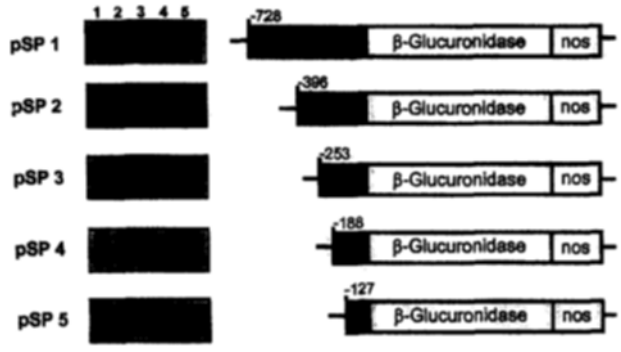

Figure 3. Northern blot analysis of chimeric S_{11} promoter/GUS fusion genes with a GUS gene probe during pollen development in each transgenic plant. Lanes 1-5 are indicated in the developmental stages of anther tissue and mature pollen; Lanes 1-4, anthers 4-1 days before flowering; lane 5, mature pollen.

expression was detected in leaves, stem, and root tissues (data not shown). An unexpected finding revealed in this study was that the GUS transgene was expressed in mature pollen and developing anther tissues during flower development. The expression pattern of GUS transgene in pollen and anther tissues was almost the same as the results reported by Chung et al. (1999).

The chromogenic reaction of GUS gene in anthers was performed with materials collected at one day before flowering. Anthers were sectioned in both horizontal and vertical directions (Fig. 4). The GUS activities appeared in the inner tissue of the anther such as vascular bundle (V) connected with anther, filament, and flower (Fig. 4B). In the endothecium (E) and stomium (St), tissues which related to dehiscence of pollen and pollen grains were detected to express the GUS gene (Fig. 4, A and B).

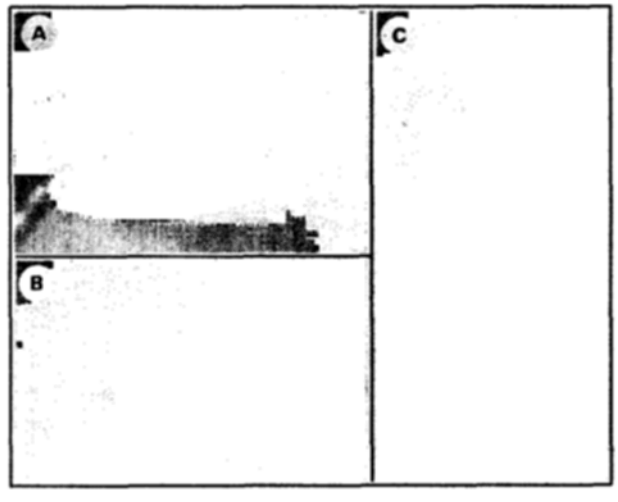

Figure 4. The expression pattern of GUS gene fused with 127 bp of S_{11} promoter region (pSP 5) in the style and anther at one day before flowering (DBF). **A**, anther tissue at 1 DBF; **B**, horizontal section; **C**, vertical section; **PG**, pollen grain; **V**, vascular bundle; **E**, epidermis; **En**, endothecium; **St**, stomium.

DISCUSSION

To study how *S* RNase genes are expressed and regulated in the floral tissues of *L. peruvianum*, sequences of the promoter region were analyzed with the S_{11} and S_{12} genomic genes, which are associated with gametophytic SI of *L. peruvianum*. Five chimeric constructs were transformed into *N. tabacum*. The expression level was similar in the style tissue of 5 GUS chimeric constructs as confirmed by Northen blot analysis (Fig. 2). This result indicates that only 127bp of *S* RNase gene promoter can control the tissue-specific expression in the style tissue.

The nature of the predicted pollen *S* allele receptor or inhibitor remains unclear. Although previous proposed models of SI indicated that the pollen and style products of the *S* locus were identical (de Nettancourt, 1977), expression of the style-expressed *S* locus sequences have not been observed in either mature pollen or in germinating pollen tubes (Kao and Huang, 1994; Dodd et al., 1996). All of five chimeric constructs showed GUS activity in the style and developing anther tissues. Anther tissues dissected vertically and horizontally showed GUS activity in the epidermis, endothecium, stomium, and vascular bundle tissues. Interestingly, GUS activity was detected in the mature pollen. To date, no expression of the pollen-expressed *S* RNase gene has been found in other Solanceous plants (Dodd et al., 1996; Chung et al., 1999) (Fig. 4, B and C).

Several models have been proposed to understand the mechanism of SI. Among these models, Thomson and Kirch (1992) proposed that the pollen component may act as an *S* RNase inhibitor or repressor to distinguish self- or nonself-pollen. According to McClure et al. (1990), that *S* RNase attacks rRNAs in self-pollen rejection raised the question of whether or not *S* RNase can enter the self-pollen tube. Another hypothesis was suggested by Lewis (1960) that the genetic structure of the *S* locus is composed of three parts with different functions. A specific part in the *S* locus may be responsible for determination of allelic specificity in the pollen and style. The active part in the *S* locus may be responsible for determination of

allelic specificity in the pollen and style. The active part in the pollen and style may each contain the specific product (Lewis, 1960). This model was based on mutants for SI in which the product of the active parts in the style have been shown to be *S* RNase. But the active part in pollen has not been identified at the molecular level (Chung and Shin, 1997). In this study, we demonstrated that the *S* RNase gene promoter from *L. peruvianum* was expressed in both the style and developing pollen tissues in transgenic tobacco plants using reverse genetics. The expression pattern of the GUS transgene in developing pollen obtained in this study indicates that SI of *L. peruvianum* occurs by an interaction of *S* RNase gene expressed both in pollen and style tissues.

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