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Expression Pattern of *S* RNase Gene Promoter in Various Floral Tissues of *Lycopersicon peruvianum*

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일시적 발현을 통한 토마토 *S* RNase gene promoter의 발현 양상

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To understand the tissue specific expression pattern of *S* RNase genes associated with self-incompatibility in *L. peruvianum*, two promoter regions of S_{11} and S_{12} RNase genes were compared. Homologous sequences between two *S* RNase gene promoters were found within 300 bp upstream of transcription start site. Moreover, short direct repeat sequences within S_{11} RNase gene promoter existed in the vicinity of 350-500 bp upstream of transcription start site. To identify whether the unique promoter sequences of S_{11} RNase gene confer the tissue specific expression, six deletion fragments for S_{11} genomic gene promoter constructed by PCR were fused to β-glucuronidase gene, and introduced into various tissues of *L. peruvianum* by microprojectile bombardment. Transient expression assays indicated that S_{11} RNase gene promoter contained the positive and negative regulatory sequences, which can control the floral tissue-specific expression in *L. peruvianum*.

Key words: self-incompatibility, tissue specific expression, promoter, transient expression, microprojectile bombardment

Higher plants do not have the ability of moving about and choosing mating partners, so the mechanisms that prevent self-pollination are of crucial importance for mating genetic variability within plant populations (Ebert et al., 1989). One of the mechanisms is self-incompatibility (SI). SI plants maintain their offspring by outcrossing with genetically different individuals among the species (Chung et al., 1995: Ebert et al., 1989: Haring et al., 1990).

About half of the families in flowering plants have homomorphic SI (Clark and Sim, 1994: Tanksley and Loaiza-Figueroa, 1985). In many of these plants, SI is controlled by a single genetic locus known as the *S* locus (Nettancourt, 1977). There are two types of homomorphic SI: sporophytic and gametophytic. In the gametophytic SI (GSI) family, rejection for self pollen occurs when *S* genotype of haploid pollen is coincident to one of *S* genotypes of the diploid style tissue. Self-pollens grow pollen tubes into the style, but they are subsequently arrested within the upper region of the incompatible transmitting tissue (Ebert et al., 1989: Haring et al., 1990: Kao and Huang, 1994). In contrast, in the sporophytic SI (SSI) family, pollen behavior is determined by the diploid *S* genotype of the pollen-producing plant and tube arrest occurs at an early stage of germination, usually on the stigma surface (Haring et al., 1990).

S RNases and their encoding cDNAs of many plants in Solanaceous family are cosegregated with their respective *S* alleles in genetic cross (Anderson et al., 1986: Clark and Sim, 1994: Mau et al., 1986). Therefore, *S* RNases have been known as a crucial factor of the SI in the Solanaceous family (Kao and Huang, 1994: Newbigin et al., 1993: Sim, 1993). *S* RNases from *Nicotiana alata* (McClure et al., 1989), *Petunia inflata* (Ai et al., 1990), *Solanum chacoense* (Xu et al., 1990) and *L. peruvianum* (Chung et al., 1993) have

ribonuclease activity, which may cause the cytotoxic effect on self-pollen tube during growth in the style tissue (McClure et al., 1990).

One of the unique features of GSI genes is the pattern of temporal and tissue-specific expression in the pistil tissue. The pattern of temporal expression of *S* RNase genes is directly correlated with the execution of self-incompatibility response and that high levels of *S* RNase mRNA accumulated preferentially in style tissue at open flowering (Chung et al., 1993, 1994, 1998). The mRNA of *S* RNase gene accumulated in the cells of the upper part of style, stylar transmitting tract, as well as in epidermal cells of the ovary placenta in *N. alata* (Cornish et al., 1987). This tissue specific expression pattern would reflect the cellular function of *S* protein encoding *S* allele gene in those locations where it can interact with elongating pollen tubes.

Several models have been proposed to understand the mechanism of SI. The tripartite model, that the genetic structure of the *S* locus is composed of three parts with different functions, was suggested by Lewis (1949, 1960). The specificity part may be responsible for determination of allelic specificity in the pollen and pistil. The active part in pollen and pistil may each contain the specific product. This model was based on mutants for SI reaction in pollen and pistil. According to recent knowledge on GSI, the product of the active part in pistil have been shown to be *S* RNase. The active part in pollen has not been identified at the molecular level. Another model has been suggested by Thompson and Kirch (1992), who proposed that the pollen component may act either as an *S* RNase inhibitor or receptor to distinguish self-or nonself-pollen. In previous models of self-incompatibility, the pollen and style products of the *S* locus were predicted to be identical (de Nettancourt, 1977). However, the *S* RNase gene expressed in style has not been observed in either mature pollen or in germinating pollen tubes (Haring et al., 1990: Chung et al., 1993, 1994). No detection of pollen expressed in *S* RNase gene could result from insufficient sensitivity for detecting rare mRNAs in mature pollen and developing pollen.

Three homologous sequences between the promoter regions of two *S* allele genes were in the vicinity of 300 base-pairs upstream from transcriptional start site (Chung et al., 1995). Those sequences might be involved in controlling the expression of *S* RNase gene in the style tissue of *L. peruvianum*.

To determine the ability of isolated *S* RNase gene promoter sequences to direct organ-specific gene expression, six deletion constructs were generated by PCR using S_{11} genomic DNA. Deletion constructs fused with β-glucuronidase (GUS) gene were introduced into various tissues of *L. peruvianum* using microprojectile bombardment. The activity of deleted S_{11} RNase gene promoter in various tissues was examined by GUS assays.

MATERIALS AND METHODS

Plant Materials

The origin of $S_{11}S_{12}$ plants of *L. peruvianum* used in this study has previously been described (Chung et al., 1994, 1995). Plants were grown on soil in the greenhouse. When they flowered, various tissues were collected and used.

PCR primers and Reaction Conditions

To construct recombinant plasmids with promoter region of S_{11} RNase genomic gene (Chung, 1997), six forward primers and one reverse primer involving two restriction enzyme sites (*Hin*dIII and *Sal*I) were designed as follows:

Forward-1 : 5′-TCAAGCTTGTCGACATAAATCATAT-3′
Forward-2 : 5′-TCAAGCTTGTAAAGATATTGATGAT-3′
Forward-3 : 5′-TCAAGCTTAAAGAACACAATCATAA-3′
Forward-4 : 5′-TCAAGCTTTAATTATTTAATAATAT3′
Forward-5 : 5′-TCAAGCTTGTACACCATAAGACTAG-3′
Forward-6 : 5′-TCAAGCTTTACTAATTATCACATGG-3′
Reverse : 5′-TCGTCGACTCTTTAGTCTGAAAAGC-3′

The reaction mixture for the amplification of plasmid DNA corresponding to S_{11} RNase gene promoter contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (W/V) gelatin, 0.2 mM of each dNTP, 2.5U AmpliTaq DNA polymerase (Promega), 0.5 μg of plasmid DNA and each 50 pmol of two appropriate primers in a 50 μL reaction volume. The amplification was performed for 35 cycles through a regime of 1 min denaturation at 94 , followed by 2 min of annealing at 45 and 2.5 min primer extension at 72 , using a DNA Thermal Cycler 2400 (Perkin-Elmer). Products were electrophoresed in an 1.5% agarose gel and the fragments were eluted using Gene-Clean (Promega).

Construction of deletion mutants from S_{11} RNase gene promoter

Mutant fragments deleted from promoter region of S_{11}

RNase gene were used to examine the expression pattern of recombinant mutants. Deletion mutants amplified by PCR were double digested with *Hin*dIII and *Sal*I, and then ligated into the unique site of *Hin*dIII-*Sal*I double digested pBI101 which is plant expression vector (Jefferson et al., 1987). Six deletion fragments fused with GUS gene were transferred into Escherichia coli DH5α.

Particle Gun Bombardments

Six plasmid DNAs (3 μg) were precipitated onto tungsten particles (1.1 mm diameter) with 2.5M $CaCl_2$, 0.1M spermidine. Microprojectile solution(10 μL) was loaded on silicon rubber 'carrier' (8 mm diameter). Materials (leaf, sepal, petal and pistil) were excised from whole plant growing in pot and placed on MS agar plate. After drying microprojectile solution, microprojectiles were bombarded to the materials (Kwon et al., 1996).

Histochemical Assay of GUS Activity

After bombardment, plant materials were incubated in the culture room at 28 for 2 days under light condition. To examine the expression of GUS gene, bombarded materials and control materials were soaked in histochemical reagent (2mM 5-bromo 4-chloro 3-indolylglucuronide) and incubated at 37 for 7 hours. After staining, they were rinsed four times with 70% ethanol at 65 and photographed with the actual microscope (Zeiss model stemi 2000C).

RESULTS

Construction of deletion fragments

To determine the regions conferring a tissue specificity in *S* RNase gene promoter, S_{11} and S_{12} genes were analyzed by comparing their DNA sequences. The result showed that three homologous sequences, which might control the expression of tissue specificity, were located around -300 bp from transcription start site (Figure 1). Direct repeat sequences were also found in the vicinity of -500 to -350 bp from trascription start site both S_{11} and S_{12} RNase genes (Figure 1).

For further investigation of these unique sequences in the tissue specific expression of S_{11} gene sequence, various deletion fragments containing homologous sequences and repeat

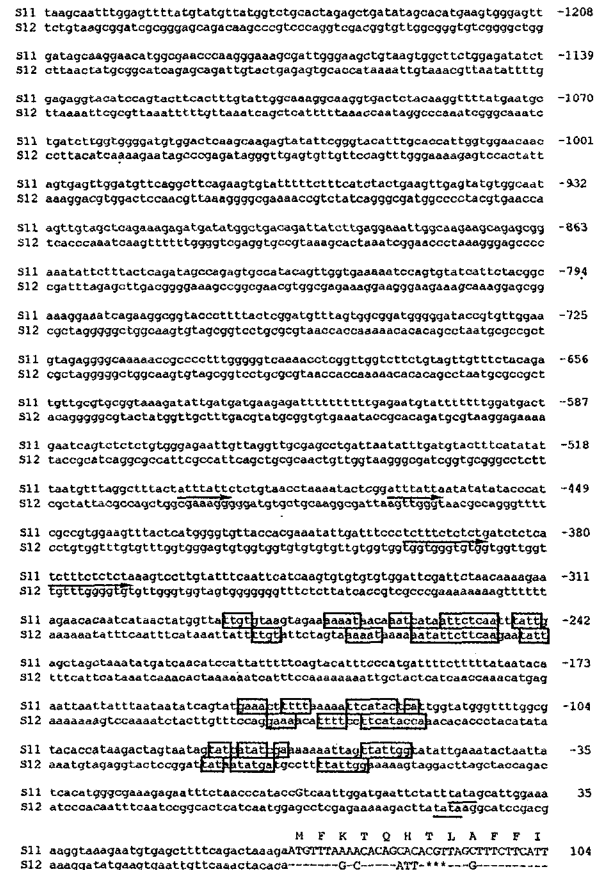

Figure 1. Comparison of the promoter sequences of the S_{11} (S_{11}) and S_{12} (S_{12}) genomic clones. Upstream sequences from ATG in the S_{11} and S_{12} genes are indicated by lowercase letters. The site of initiation of transcription of the S_{11} gene is indicated by uppercase letter in the upstream sequence. The homologous sequences in 5' upstream regions of the two *S* RNase gene promoters are enclosed in boxes. The two direct repeat sequences for S_{11} RNase gene promoter is indicated by arrows and putative TATA boxes are underlined.

sequences between S_{11} and S_{12} promoter regions were amplified with S_{11} RNase gene promoter by PCR. To select clones containing S_{11} RNase gene promoter sequences correctly, DNA sequence analysis of deletion mutants was performed using automatic sequencer (Pharmacia biotech). For cloning into the plant transformation vector (pBI101), two unique restriction enzyme sites, *Hin*dIII and *Sal*I, were added in the region of 5' and 3' end in PCR primers, respectively. PCR primer sequences were described in materials and methods.

As a result, various deletion fragments containing different lengths of S_{11} RNase gene promoter fused with the GUS gene were generated (Figure 2).

These six chimeric constructs were designated as pSP1 to 6, and were used as donor for particle gun bombardment. Deletion fragments of 396 (pSP3), 253 (pSP4) and 188 (pSP

5) base pairs in length were designed as according to homologous sequences in 5' upstream region of two *S* RNase gene (Chung et al., 1995). Fragment of 2028 (pSP1) bp was amplified the whole promoter sequence of S_{11} genomic gene. In addition, 728 (pSP2) and 127 (pSP6) bp fragments were randomly chosen in promoter region of S_{11} genomic gene.

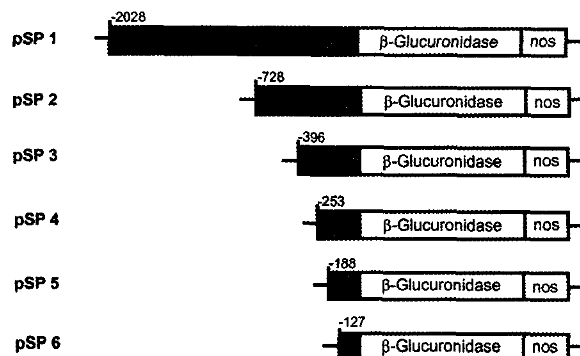

Figure 2. Six chimeric constructs of S_{11} RNase gene promoter sequence fused with GUS report gene. Arrowheads indicate the position of the transcription start site (Chung et al., 1995). The labels of the individual constructs designate the amount of S_{11} promoter sequence upstream of transcription start site.

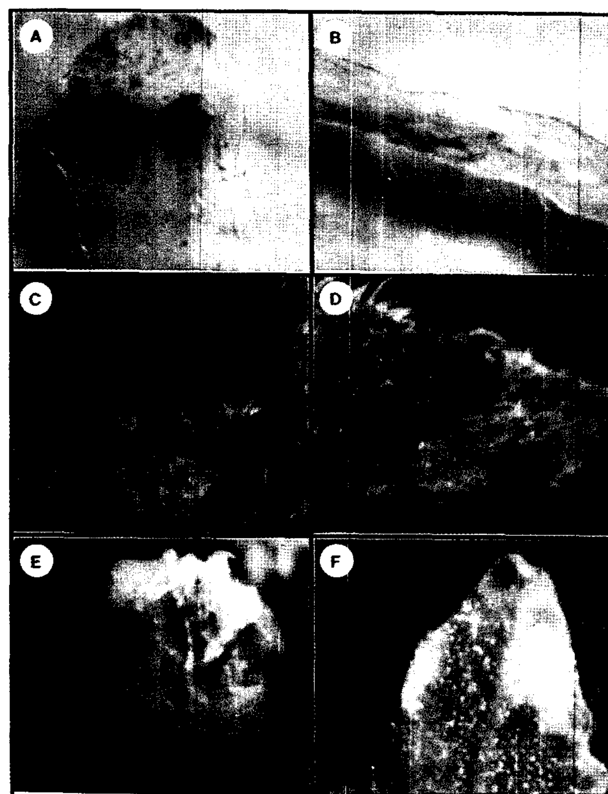

Figure 3. Histochemical analysis of GUS activity in various tissues of *L. peruvianum*. GUS activity is shown by blue staining. Cross-sections of stigma (A) and transmitting tissue (B), which are self- or nonself pollen tube elongation tissues. Petal (C), sepal (D), ovary (E) and leaf (F) tissues.

GUS activity in pistil tissue

Six chimeric constructs introduced into the various tissues of $S_{11}S_{12}$ genotype plant (*L. peruvianum*) were analyzed for their role in gene expression. As shown in (Figure 3), pistil tissue containing stigma and style showed high GUS gene expression as compared to other tissues (Figure 3A and B). However, no difference in GUS gene expression was detected among 6 chimeric constructs in the pistil tissue (Table 1), indicating that only 127 (pSP 6) upstream sequence of S_{11} RNase gene is enough to confer *S* RNase expression in the pistil tissue of *L. peruvianum*.

Histochemical analysis of S_{11} RNase gene promoter/GUS expression in various tissues

Although the level of GUS gene expression is different, all six chimeric constructs showed the expression in stigma and style tissues. To examine the expression pattern in stigma and transmitting tissues of style, which interact with self or non-self pollen, the tissues were dissected longitudinally and analyzed for GUS gene expression as shown in Figure 3A and B. Stigma sections showed intense staining in the papillar cells, in which dehydrogenated pollen penetrate, and lower activity levels in the subepidermal cells (Figure 3A). GUS staining was observed in the central cells of the solid style within a zone defined cytologically as the transmitting tract, which is a path of pollen tube growth toward ovule (Figure 3B).

To determine whether expression of six different chimeric structures restricted only in the pistil tissue, petal, sepal, leaf, and ovary tissues were bombarded with these six constructs. As shown in (Figure 3C, D, E and F), S_{11} RNase gene promoter seemed to be involved in the specific sequences for tissue specific expression in these tissues. The different expression pattern of pSP1 to 6 deletion constructs is shown in the (Table 1). No expression was observed in leaves bombarded with all deletion fragment constructs of S_{11} RNase gene promoter. However, GUS gene expressed in the petal, sepal, and ovary tissues as shown in Figure 3C, D and E: the constructs pSP 5 and pSP 6 expressed in the petal tissue but others did not. In case of sepal tissue, only pSP1 and pSP2 constructs expressed GUS gene. GUS gene expression in the ovary tissue was detected only when pSP 6 construct was introduced.

Table 1. Histochemical analysis of various tomato tissues transformed with six S_{11} promoter sequences fuesd with GUS reporter gene by particle gun bombardment.

Chimeric construct	GUS activity[a]					
	Pistil	Leaf	Sepal	Petal	Ovary	Pollen
pSp_1(2028 bp)	+	-	+	-	-	nt
pSp_2(728 bp)	+	-	+	-	-	nt
pSp_3(396 bp)	+	-	-	-	-	nt
pSp_4(253 bp)	+	-	-	-	-	nt
pSp_5(188 bp)	+	-	-	+	-	nt
pSp_6(127 bp)	+	-	-	+	+	nt

[a]GUS expression detected(+), No GUS expression detected(-), Not tested(nt)

DISCUSSION

To determine regulatory sequences of *S* RNase promoter responsible for the specific expression of style tissue in *L. peruvianum*, we have now characterized the pattern of tissue specific expression of deletion fragments 2028 (pSP 1), 728 (pSP 2), 396 (pSP 3), 253 (pSP 4), 188 (pSP 5) and 127 (pSP 6) base pair in length fused with GUS gene. In general, promoter elements of plant genes are found within a few hundred bases upstream of transcription start sites (Benfey and Chua, 1989). In case of self-incompatibility gene, a common motif 1 (ACATAAT-ATTATGT), which forms an inverted repeat, was found in the upstream sequence from transcription start sites between *S* RNase genes of *S. tuberosum* (-146 in the S_2 allele) and *P. inflata* (-111 in the S_1 allele and -603 in the S_3 allele), although the position of the motifs in the *S* RNases of two plants is quite different (Kaufman et al., 1991: Coleman and Kao, 1992). However, motif 1 was not found in either the S_{11} or S_{12} allele of *L. peruvianum*. Sequence comparisons of the upstream regions in the S_{11} and S_{12} allele genomic fragments revealed that they have relatively heterologous sequences, but three different motifs and direct repeat sequences were found within 300 and 350-500 bp upstream from the transcription start site of the S_{11} and S_{12} allele genes, respectively (Figure 1).

Chimeric constructs of pSP 3 to 5/GUS fusion gene were designed according to three different motifs of S_{11} RNase gene promoter that might confer tissue specificity of pistil tissue. Regulatory sequences can be divided into two classes according to whether they confer qualitative or quantitative expression. Regulatory sequence conferring a qualitative expression pattern of organ-specific were identified using the histochemical staining for transient expression after bombardment to various tissues with pSP 1 to 6/GUS fusion construct. However, quantitative regulation of the S_{11} RNase gene expression can not be interpreted because the result obtained by bombardment is variable.

Various tissues bombarded with pSP1 to 6/GUS fusion constructs were investigated for the difference of GUS expression. Of pSP 1 to 6/GUS fusion genes, pSP 1 and 2/GUS fusion constructs only expressed in the sepal tissue, while pSP 5 and 6/GUS fusion constructs were observed to express only in the petal tissue. In addition, pSP 6/GUS fusion construct expressed in the ovary tissue, too. In the pistil tissue, all of tested constructs expressed GUS gene with similar pattern. It is noteworthy that the common sequence contained in all these constructs is TATA box. These results indicated that the negative regulatory sequence of petal tissue may be located at -188 to -2028 bp on the S_{11} RNase gene promoter, while the positive regulatory sequence conferring S_{11} RNase gene expression in the sepal may be located at -396 to -2028 base pair on the S_{11} RNase gene promoter. Owing to the physical properties of the pollen, investigation for the expression of pSP1-6/GUS fusion construct using particle gun bombardment was not performed. SI occurs as the result of interaction between S_{11} RNase expression in the pistil and the pollen factors (Haring et al., 1990: Kao and Huang, 1994: Newbigin et al., 1993). So, it is important to investigate the expression of the *S* RNase gene in the pollen. To identify the expression pattern of various chimeric structures in pollen tissues and to investigate the quantitative regulation of *S* RNase gene, our studies are in progress to demonstrate *S* RNase gene expression during pollen development using transgenic plants (*N. tabacum*) with pSP1-6/GUS fusion constructs. Finally, determination of the regulatory elements controlling the tissue specific expression of the *S* RNase gene promoter will be investigated soon. Characterization of the *S* gene promoter in pollen tissue will be promising to understand the system of gametophytic self-incompatibility.

적 요

야생종 토마토의 자가불화합성에 관여하는 *S* RNase 유전자의 조직특이적 발현 양상을 조사하기 위하여 S_{11} 및 S_{12} allele에 속하는 RNase 유전자의 promoter영역에 대한 염기서열을 비교 분석한 결과, 전사개시점에서 상류측으로 350-500bp사이에서 양쪽 allele의 promoter간에 상동성을 나타내는 3부분과 direct repeat sequence를 발견하였다. Promoter영역에서 이러한 부분이 *S* RNase 유전자가 화주특이적으로 발현하는데 영향을 줄것으로 예상하고, 이들 영역을 중심으

로 6종류의 deletion fragment를 만들어 GUS 유전자에 연결하여, 토마토의 생식조직에 microprojectile bombardment를 수행하였다. 그 결과 토마토 자가불화합성에 관여하는 *S* RNase 유전자의 promoter는 TATA box를 포함한 127 bp만으로도 화주조직 특이적 발현을 조절할수 있었다. 또한 *S* RNase 유전자의 promoter영역내에는 토마토 화변, 자방과 심피조직들에서 negative 혹은 positive로 유전자의 발현을 유도하는 부분이 발견되었다.

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