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Cloning, characterization and promoter analysis of *S*-RNase gene promoter from Chinese pear (*Pyrus pyrifolia*)

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ABSTRACT

The 5′-flanking region of the S_{12}-, S_{13}-, S_{21}-RNase with a length of 854 bp, 1448 bp and 1137 bp were successfully isolated by TAIL-PCR from genomic DNA from 'Jinhua', 'Maogong' (*Pyrus pyrifolia*) and 'Yali' (*Pyrus bretschneideri*) genomic DNA. Sequence alignment and analysis of S_{13}-, S_{12}-, S_{21}-*RNase* gene promoter sequences with S_2-, S_3-, S_4-, S_5-*RNase* 5′-flanking sequences indicated that a homology region of about 240 bp exists in the regions just upstream of the putative TATA boxes of the seven Chinese/Japanese pear S-RNase genes. Phylogenetic tree suggests that the homology region between the Chinese/Japanese pear and apple *S-RNase* gene promoter regions reflects the divergence of *S-RNase* gene was formed before the differentiation of subfamilies. Full length and a series of 5′-deletion fragments-GUS fusions were constructed and introduced into *Arabidopsis thaliana* plants. GUS activity were detected in S_{12}-pro-(1 to 5)-GUS-pBll01.2 transgenic pistils and progressively decreased from S_{12}-pro-1-GUS-pBI l01.2 to S_{12}-pro-5-GUS-pBll01.2. No GUS activity was detected in S_{12}-pro-6-GUS-pBll01.2 transgenic pistil and other tissues of non-transformants and all transgenic plants. The result suggested S_{12}-*RNase* promoter is pistil specific expression promoter.

1. Introduction

Self-incompatibility (SI) is a mechanism that prevents inbreeding and promotes outbreeding in flowering plants by enabling the pistil to reject pollen from genetically related individuals, especially in pear. Chinese pear, a member of the Rosaceae, has a gametophytic self-incompatibility (GSI) controlled by a single locus (*S*-locus) with multiple alleles called the *S*-locus (de Nettancourt, 1997) as one type of SI. *S*-allele-associated stylar glycoproteins with RNase activity, termed *S*-RNases, have been identified by protein analysis (Ishimizu et al., 1996; Sassa et al., 1992), and have been proved to be responsible for GSI in pear.

The expression of *S*-RNase is specific to the pistil style and is regulated during flower development (Ishimizu et al., 1996; Sassa et al., 1993), suggesting involvement of *cis*-regulatory element(s) in the promoter and *trans*-activator(s). Attempts have been made to indentify the *cis*-regulatory elements for the pistil-specific expression in the *S*-RNase promoter in the Solanaceae and Rosaceae. But the attempts of solanaceous *S*-RNase genes have been difficult because these regions are highly heterogeneous even in the putative promoter (Chung et al., 1995; Coleman and Kao, 1992; Kaufmann et al., 1991; Matton et al., 1995). Furthermore the rosaceous *S*-RNases having an approximately 200 bp region, which may contain *cis*-elements, were found upstream of the putative TATA boxes in the three Chinese pear *S*-RNases ($P.pS_{12}$-, $P.pS_{13}$- and $P.bS_{21}$-RNase) and four Japanese pear *S*-RNase ($P.pS_2$-, $P.pS_3$-, $P.pS_4$- and $P.pS_5$-RNase) and two apple *S*-RNases (MdS_9- and MdS_f-RNase), but not in the apple S_2-RNase gene (Ushijima et al., 1998a).

In this study, three *S*-RNases (S_{12}-, S_{13}-, and S_{21}-RNase) promoters were amplified by TAIL-PCR technique from Chinese pear ['Jinhua' ($S_{13}S_{18}$, *Pyrus pyrifolia*, *P.p*), 'Maogong' ($S_{12}S_{13}$, *P.p*) and 'Yali' (S_1S_{21}, *Pyrus bretschneideri*, *P.b*)]. Comparison and analysis of these rosaceous *S-RNases* promoters finds an approximately 240 bp region upstream of the putative TATA boxes of the eight apple/pear *S-RNases*, but it is 40 bp shorter in the $P.pS_2$-RNase gene (Ushijima et al., 1998a). This homologous 240 bp region (box 1, from −107 to −346, the numbering is based on the S_{12}-*RNase* gene, the same below) may contain *cis*-regulatory elements. The regions upstream of box 1 are heterogeneous among the pear *S-RNases* although a further 125 bp homologous region (box 2, from −357 to −482) is present just upstream of the box 1 between S_3- and S_5-*RNase* genes (Norioka et al., 2001). Then introduced GUS genes driven by 6 sequential deletions of $P.pS_{12}$-*RNase* promoter into *Arabidopsis thaliana* Columbia 0 using *Agrobacterium*-mediated *floral dip* transformation. Analysis of GUS expression in the transgenic *A. thaliana* plants revealed the localization of the *cis*-regulatory elements in $P.pS_{12}$-*RNase* promoter region.

Abbreviations: SI, Self-incompatibility; GSI, Gametophytic self-incompatibility; *P.p*, *Pyrus pyrifolia*; *P.b*, *Pyrus bretschneideri*; *Md*, *Malus*×*domestica*; *N. alata*, *Nicotiana alata*; bp, Base pair; kb, Kilobase; TAIL-PCR, Thermal asymmetric interlaced PCR; AD, Arbitrary degenerate; BLAST, Basic local alignment search tool; NCBI, National Center for Biotechnology Information; MS, Murashige and Skoog; X-Gluc, 5-bromo-4-chloro-3-indyle-β-D-glucuronide.

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2. Materials and methods

2.1. Plant materials, growth conditions and extractions of genomic DNA

Young leaves of three Chinese pear 'Jinhua' ($S_{13}S_{18}$, *P.p*) and 'Maogong' ($S_{12}S_{13}$, *P.p*) and 'Yali' (S_1S_{21}, *P.b*) cultivars were collected in spring at the Zhengzhou Fruit Institute, Chinese Academy of Agricultural Sciences, Zhengzhou, Henan, China. The leaves were stored at −80 °C before being used and genomic DNA was isolated from 100 to 200 mg of pear leaves using Plant Genome DNA Extraction Kit of Beijing Sunbiotech Co. Ltd. The DNA concentration was measured by gel electrophoresis using spectrophotometer. Then the DNA extract was kept at −20 °C until use. The genomic DNA was used as templates for TAIL-PCR.

A. thaliana ecotype Columbia 0 plants were grown in a constant-temperature room at 22 °C under 16 h light/8 h dark cycles.

2.2. TAIL-PCR and Verification PCR of the 5′-flanking regions of Pyrus S-RNase genes

The TAIL-PCR primers GSP1, GSP2 and GSP3 (Table 1) were designed in nested positions based on the reserved 5′-flanking regions of *S-RNase* coding sequences in combination with 6 arbitrary degenerate (AD, Table 1) primers. TAIL-PCR protocol was performed according to the method described by Liu and Whittier (1995). The target products were identified by expected size differences and purified using a Gel Extraction Kit (Ambiogen, China) then sequenced in both directions by China Nanjing GenScript, Corporation.

Three new specific primers FP4, FP5 and FP6 (Table 1) were designed according to the sequences which we obtained by TAIL-PCR for the purpose of verifying the PCR fragments which indeed represent in the upstream of *S-RNases*. And then amplifications were carried out using the primers FP4/5/6 and P2 (Table 1).

The DNA sequences obtained were analyzed using the tools provided by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Sequence analysis and construction of phylogenetic tree of Rosaceae S-RNase gene promoters

All the sequence data were analyzed using BLAST at the National Center for Biotechnology Information (NCBI), PLACE (http://www.dna.affrc.go.jp/PLACE/), PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and GENDOC (http://www.nrbsc.org/gfx/genedoc/), Vector NTI10.3 (http://www.invitrogen.com/site/us/en/home/LINNEA-Online-Guides/LINNEA-Communities/Vector-NTI-Community/vector-nti-software.html), Clustal X 2.1 software (http://www.clustal.org/clustal2/), MEGA4 (http://www.megasoftware.net/mega.html), Phylip (http://psc.edu/general/software/packages/phylip/).

2.4. S_{12}-RNase promoter deletion-GUS vector constructs

A 1564 bp fragment obtained by PCR using primer pair GSP3/FP7 (Table 1) and 'Maogong' genomic DNA as the template containing S_{12}-*RNase* promoter region (−1448 to −1) and truncated coding region (+1 to +116) was ligated into T vector (TaKaRa Japan) and used as template for making sequential deletions of S_{12}-*RNase* promoter. 6 forward primers with *Sal* I site and a reverse primer with *Xba* I site were designed and then the sequential deletions of S_{12}-*RNase* promoter were amplified from the 1564 bp fragments using an Expand high-fidelity PCR system with each forward primers and PpS_{12}pro-RV primer. PCR amplification was carried out for 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 2 min with a final extension of 7 min. Six amplified fragments were digested with *Sal* I/*Xba* I and inserted into *Sal* I/*Xba* I site upstream of GUS gene

Table 1
Primers used for the study.

Name	Primer sequence	Position	Reference	Note
TAIL-PCR primers				
GSP1	5′-TATTTGTTCAAGAGCGAGGC-3′	−27 to −7		
GSP2	5′-CCGTGGACGAAGACAATATT-3′	+54 to +73		
GSP3	5′-GCCGGCTGATATTGCTGCGTAAATT-3′	+92 to +116		
AD1	5′-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3		Liu et al. (1995)	
AD2	5′-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3′			
AD3	5′-(A/T)GTGNAG(A/T)ANCANAGA-3′			
AD4	5′-AG(A/T)GNAG(A/T)ANCA(A/T)AGG-3′		Liu and Whittier (1995)	
AD5	5′-TG(A/T)GNAG(G/C)ANCA(G/C)AGA-3′			
AD6	5′-(G/C)TTGNTA(G/C)TNCTNTGC-3′		Tsugeki et al. (1996)	
Verification PCR				
FP4		5′-ACTACACAGGAGAACTAACA-3′		
FP5	5′-TGTTCACCCCAAATGTAGAC-3′			
FP6		5′-TGAATCGTTGTGGTCATCTC-3′		
P2	5′-AC(A/G)TTCGGCCAAATAATT-3′		Yamamoto et al. (2002)	
PCR for the template making sequential deletions of S_{12}-RNase promoter				
FP7	5′-TTGGGCAGGACCAGAGAGAGACTAC-3′			
S_{12}-RNase promoter deletion-GUS vector constructs				
PpS_{12}pro-1	5′-ATTCGTCGACTTGGGCAGGACC-3′	−1448to −1437		*Xba* I
PpS_{12}pro-2	5′-ACGCGTCGACGGGTTACATTACATCC-3′	−1138to −1123		
PpS_{12}pro-3	5′-GCAAGTCGACCCTTCTCCACCCAA-3′	−865 to −852		
PpS_{12}pro-4	5′-ACGCGTCGACCCCGTCTATGCTCAAG-3′	−505 to −490		
PpS_{12}pro-5	5′-CCACGTCGACAGAAACACAACA-3′	−297 to −286		
PpS_{12}pro-6	5′-GAGCGTCGACATCAACTCAGGGC-3′	−257 to −245		
PpS_{12}pro-RV	5′-CGTCTAGACGTCCCCATTGAATAA-3′	+15 to −7		*Xba* I
Verification PCR for the linkage of PpS_{12}pro-(1–6)-GUS fusions				
PpS_{12}GUS-F	5-CAGGGCATTGCACATGACTA-3			
PpS_{12}GUS-R	5-TACGGCGTGACATCGGCTTC-3			