Scientia Horticulturae 207 (2016) 218–224

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Cloning and characterization of an *S-RNase* gene in *Camellia sinensis*

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ARTICLE INFO

Article history:
Received 7 April 2016
Received in revised form 2 June 2016
Accepted 3 June 2016
Available online 16 June 2016

Keywords:
Camellia sinensis
Tea
Self-incompatibility
CsS-RNase
S-RNase
Genetic linkage map

ABSTRACT

Self-incompatibility (SI) prevents inbreeding depression in angiosperms. *Camellia sinensis* is an important cash crop, but breeding improvements and genetic studies of the plant are hindered by SI. However, the SI mechanism in *C. sinensis* remains unclear. In this study, a putative *S-RNase* gene (KU852488) was cloned from *C. sinensis*. The full-length cDNA of *CsS-RNase* is 1121 bp, which encodes 238 amino acids. It shares the closest relationship with an *S-RNase* gene (ADA67883.1), which was cloned from a self-incompatibility *Citrus reticulata* cultivar 'Wuzishatangju'. The expression level of *CsS-RNase* in the styles were 3–259 ('Fuding Dabaicha') and 5.6–119 ('Zhongcha108') times higher than the other tissues, for example petals, pollen grains, filaments and buds. And its expression rose in self-pollinated styles with 24 h earlier than cross-pollinated styles. The genotypes of *CsS-RNase* in 10 cultivars and one breeding line of *C. sinensis* were analyzed. Totally, 11 polymorphic amino acid residues were identified. A single nucleotide polymorphism (SNP) marker of *CsS-RNase* was developed. Finally, the *CsS-RNase* was mapped onto a reference genetic linkage map of tea plant.

1. Introduction

Self-incompatibility (SI) is a critical mechanism to prevent inbreeding depression in flowering plants (Wheeler et al., 2009). SI occurs in various angiosperms; however, the molecular mechanism has been determined in only small number of plants (Gibbs, 2014). On the basis of the genetic mechanism, SI can be classified into two major systems: sporophytic self-incompatibility (SSI) and gametophytic self-incompatibility (GSI). GSI is thought to be the most widespread SI type and is primarily determined by a single multi-allelic gene of the *S-locus,* including a female determinant *S-RNase* and a male determinant *S locus F-box* (*SFB*/*SLF*) (McClure, 2006). In Rosaceae, Solanaceae and Plantaginaceae, S-RNase recognizes the SFB/SLF, resulting in the rejection of self-pollen tubes in the styles (Murfett et al., 1994; Sijacic et al., 2004; McClure, 2006). The *S-RNase* gene was first cloned in *Nicotiana alata* and encodes a 32 kD glycoprotein with ribonuclease activity (Anderson et al., 1986). Loss- and gain-of-function experiments revealed that S-RNase mediates self-pollen tube rejection in *Petunia inflate* and *N. alata* (Lee et al., 1994; Murfett et al., 1994). To date, *S-RNases* have been isolated and identified in several plants, such as *Malus pumila*, *Petunia hybrid*, *Pyrus serotina*, *Prunus avium*, *Prunus armeniaca* and *Citrus reticulata* (Murfett et al., 1994; Broothaerts et al., 1995; Xue et al., 1996; Ushijima et al., 1998; Castillo et al., 2002; Miao et al., 2011).

The tea plant (*Camellia sinensis*) is an important beverage crop in the world (Wei et al., 2014). Due to self-sterility and long-term hybridization, *C. sinensis* is highly heterogeneous, which inhibits genetic improvements and breeding research (Tan et al., 2013). Therefore, it is essential to clarify the molecular mechanisms of SI in *C. sinensis*. Nevertheless, several attempts have been made. Tomimoto et al. (1999) isolated a pistil-specific

Abbreviations: SI, self-incompatibility; SSI, sporophytic self-incompatibility; GSI, gametophytic self-incompatibility; SFB/SLF, S locus F-box; CsS-RNase, *Camellia sinensis* S-RNase; SP, self-pollination; CP, cross-pollination; EB, ethidium bromide; ORF, open reading frame; SNP, single nucleotide polymorphism; dCAPS, derived cleaved amplified polymorphic sequence; RT-PCR, semi-quantitative real-time PCR; SSR, simple sequence repeat; SRK, S-receptor kinase; SCR, S-locus cysteine-rich protein; CAS, conserved amino acid; MAS, marker-assisted selection; QTL, quantitative trail loci.

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http://dx.doi.org/10.1016/j.scienta.2016.06.002

Table 1
Tea cultivars used in *CsS-RNase* genotyping.

Name	Code	Name	Code
Zhongcha108	ZC108	Longjing43	LJ43
Anji Baicha	AJBC	Fuding Dabaicha	FDDB
Zhenong117	ZN117	Yingshuang	YS
Fuyun6	FY6	Maolv	ML
Longjing Changye	LJCY	Longjing001[a]	LJ001
Zhenong139	ZN139		

[a] Indicates a breeding line.

pathogenesis-related-1 (PR-1) protein using a two-dimensional (2D) gel electrophoresis method. To explore SI-related candidate genes, Chen et al. (2011) obtained 25 differentially expressed genes from different pollination treatments of *C. sinensis* by a cDNA-AFLP method. These genes were supposed to associate with programmed cell death, signal transduction, and Ca^{2+} concentration regulation. Tan et al. (2013) revealed several pollen/pistil specific genes by transcriptome analysis. However, the molecular basis of SI in *C. sinensis* remains unclear.

Recently, six transcriptomes of self/cross-pollination styles from tea plants were sequenced and analyzed by our laboratory. A putative *S-RNase* gene (CL25983Contig1) with strong homology to RNase T2 was obtained. In this paper, the full-length *C. sinensis S-RNase* (*CsS-RNase*) was cloned and its expression profiles were detected in various tissues and at different times after self/cross-pollination treatments. After that, *CsS-RNases* were genotyped in several cultivars of *C. sinensis*. Finally, the *CsS-RNase* was mapped on the reference genetic map of *C. sinensis*. We believe that the revelation of the *CsS-RNase* gene helps to characterize the SI mechanism and will promote breeding studies and genetic research in the tea plant.

2. Materials and methods

2.1. Plant materials, RNA and DNA extraction

Tissues from petals, styles, ovaries, sepals, pollen grains, buds and leaves were collected from 'Fuding Dabaicha' (FDDB) and 'Zhongcha108' (ZC108), frozen in liquid nitrogen immediately and stored at −80 °C. The pollination combinations, including FDDB (♀) x FDDB (♂) (self-pollination, SP) and FDDB (♀) x ZC108 (♂) (cross-pollination, CP), were performed. The styles were harvested at 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h and 120 h after pollination. Total RNA was extracted using an RNAprep pure Plant Kit (Tiangen, Beijing, China).

Ten cultivars ('Longjing43', 'Zhenong117', 'Zhongcha108', 'Zhenong139', 'Anji Baicha', 'Fuyun6', 'Fuding Dabaicha', 'Longjing Changye', 'Maolv', and 'Yingshuang') from the China National Germplasm Hangzhou Tea Repository and a breeding line 'Longjing001' were used to determine the haplotypes of *Cs-SRNase* (Table 1).

An F1 segregating population (166 individuals) generated by 'Longjing43' (♀) and 'Baihaozao' (BHZ) (♂) was used to map *CsS-RNase* on a genetic linkage map. Genomic DNA was extracted from 166 individuals and 2 parents using a Plant DNA Extraction Kit (Tiangen, Beijing, China).

2.2. Cloning and analysis of CsS-RNase

The sequence of CL25983Contig1 with strong homology to RNase T2 and different expression levels between CP and SP was selected. A specific primer of C25983C1-3′-1 was designed to obtain the 3′ fragment of CL25983Contig1 using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). The target PCR product was cloned into *Escherichia coli* DH5α competent cells (Tiangen, Beijing, China) and sequenced by Thermo Fisher (Shanghai, China). A DNAstar program Editseq option (DNASTAR, Inc., Madison, Wis.) was employed to predict the open reading frame (ORF). To amplify the intact ORF, the primers CsSRNase-1F and CsSRNase-1R, located in the 5′-UTR and 3′-UTR, respectively, were designed using Primer Premier 5.0 software (Premier Biosoft Ltd., Palo Alto, CA, USA). The first strand cDNA was synthesized from the total RNA using a PrimeScript® 1st strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The reaction volume was 50 μL, containing 0.5 μL *LATaq*, 10 μL 10 × PCR buffer, 4 μL dNTP (2.5 mM), 1 μL of each primer (10 μM), 2 μL cDNA (100 ng) and 31.5 μL ddH_2O. The PCR program was as follows: 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min; and a 4 °C hold. The PCR product was examined on a 1% agarose gel stained with ethidium bromide (EB) and purified using an agarose gel DNA Purification Kit (TaKaRa, Dalian, China). Next, the target product was inserted into the pMD18-T vector (TaKaRa, Dalian, China) and transformed into DH5α competent cells. Finally, the positive recombinant plasmids were sequenced from both directions.

2.3. Expression analysis of Cs-SRNase

The expression patterns of *Cs-SRNase* were examined using a semi-quantitative reverse transcription PCR (sqRT-PCR) and a quantitative real-time PCR (qRT-RCR) method with the reference gene *GAPDH* (GE651107). The reaction volume of sqRT-PCR was 25 μL, containing 0.5 μL *LATaq*, 5 μL PCR buffer, 2 μL dNTP (2.5 mM), 0.5 μL of each primer (10 μM), 1 μL cDNA (40 ng) and 15.5 μL ddH_2O. The amplification process was as follows: 94 °C for 3 min; 28 cycles (*GAPDH*) or 30 cycles (*Cs-SRNase*) at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min; and a 4 °C hold. Three independent RT-PCR reactions were performed. The products were then separated on a 1% agarose gel stained with EB. The qRT-PCR reactions were performed using a PrimeScriptTM RT reagent qPCR Kit (TaKaRa, Dalian, China), on an ABI 7500 Real-Time PCR System (Applied Biosystems). Three biological and technical replicates were carried out for each qRT-PCR reaction. The reaction procedure was as follow: 95 °C for 30 s; 40 cycles at 95 °C for 5 s, 60 °C for 34 s; and a dissociation stage of 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s, and 60 °C for 15 s. Each reaction containing 40 ng cDNA, 5 μL SYBR Fast qPCR Mix (2 ×), 0.2 μL of each primer (10 μM), 0.2 μL ROX Reference Dye (50 ×). A $2^{-\Delta\Delta Ct}$ data analysis method was used to calculate the relative quantitation of the *Cs-RNase*.

2.4. Genotyping of Cs-SRNase

Total RNA extracted from 10 cultivars and 1 breeding line was reverse-transcribed into cDNA (Table 1). Then, the full-length *Cs-SRNases* were amplified from these samples using the primers CsSRNase-1F and CsSRNase-1R (Table 2). The PCR reaction was carried out in a 50 μL volume, containing 0.5 μL PrimeSTAR HS DNA polymerase (TaKaRa, Dalian, China), 10 μL 5 × PrimeSTAR Buffer, 4 μL dNTP (2.5 mM), 1 μL of each primer (10 μM), 2 μL cDNA (100 ng) and 31.5 μL ddH_2O. The PCR program was at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min; and a 4 °C hold. Subsequently, amplification products were sequenced from both directions. The cDNA sequences were translated into amino acids, aligned using ClustalX2 (Larkin et al., 2007) and analyzed using MEGA5.2 software (www.megasoftware.net). The domains and active sites were predicted using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Mapping Cs-SRNase on the genetic linkage map

A fragment of *CsS-RNase* was amplified from genomic DNA of 'Fuding Dabaicha' using the specific primers CsS-RNase2F and CsS-

Table 2
The primers used in this study.

Primer Code	Primer Sequence (5′–3′)	Function
C25983C1-3′-1	TCATCAAATGCCCAGTTCTACCC	3′ RACE
CsSRNase-1F	AAACAAGAAAGATGATGTCAAACCC	Full-length cDNA application
CsSRNase-1R	ACAACTAAATCGCCCACGCATA	Full-length cDNA application
rtCsSRNase-F	CTGCGATAGCCGCAACTCTT	sqRT-PCR and qRT-PCR
rtCsSRNase-R	GGAAGTAGCTGTGCTGGTCAA	sqRT-PCR and qRT-PCR
GAPDH-F	GGAAGCATCATGAACTCAAAGTGAA	Reference gene
GAPDH-R	ATCCTTCTCATTGACACCCATAACA	Reference gene
CsSRNase-2F	TGAATGGAATAAACATGGGACTTGT	SNP marker finding
CsSRNase-1R	ACAACTAAATCGCCCACGCATA	SNP marker finding
dC1F	GATGTATCCACATTGCACTCAgT	SNP detection
dC1R	AGCCAATTTTTCCCTGAGCA	SNP detection

Note: The lowercase indicates the position of a mismatched base that was used to create a restriction site (*Dra III*, 5′-CACNNNGTG-3′).

RNase1R (Table 2). The PCR product was purified and sequenced from both directions after it was transformed into DH5α competent cells (Tiangen, Beijing, China). Next, the sequences were aligned with the full-length cDNA of *CsS-RNase* using the online program Spidey (http://www.ncbi.nlm.nih.gov/spidey/). After confirming the availability of the primers, genomic DNA from 'Longjing43' and 'Baihaozao' was used to amplify the fragments of *CsS-RNase*. The PCR products were directly sequenced from both sides. Next, the sequences were aligned using the Seqman program of DNAstar software (DNASTAR, Inc., Madison, Wis.) and mined for single nucleotide polymorphism (SNP) markers following the method described by Zhang et al. (2014).

For convenient detection, the SNP was converted into a derived cleaved amplified polymorphic sequence (dCAPS) marker. The dCAPS primers were designed by the online process dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.htm) (Neff et al., 2002). A restriction site was created by the target SNP and the forward primer as described by Shahinnia and Sayed-Tabatabaei (2009) and Zhang et al. (2012). Subsequently, the dCAPS primers were used to amplify the target bands from the F1 segregating population. The PCR products were digested by *Dra III* (Toyobo Life Science, Shanghai, China), following the manufacturer's instructions. After that step, the samples were separated by silver staining polyacrylamide gel electrophoresis. The genotyping results were analyzed with the segregating data of 483 SSR markers in the same mapping population (Tan et al., 2016) using JoinMap 4.0 (van Ooijen, 2006).

3. Results

3.1. Cloning the full-length cDNA of Cs-SRNase

To isolate the full-length cDNA of *CsS-RNase* from 'Fuding Dabaicha', 3′ RACE technology was employed to amplify the 3′ fragment (Fig. 1A). The PCR products of approximately 400 bp and 700 bp were purified and sequenced after transformation into *E. coli* DH5α competent cells. As a result, the smaller band was the target PCR product and the larger one was nonspecific amplification. Therefore, a target fragment of 363 bp was isolated (Fig. 1A). After alignment with the CL25983Contig1 sequence, the full-length *Cs-SRNase* was obtained. The cDNA was 1121 bp, including an intact ORF of 717 bp, which encoded 238 amino acids (Fig. 1B, Fig. 2). Then the *Cs-SRNase* was submitted to GenBank under the accession number KU852488. A phylogenetic tree was constructed using amino acid sequences of *CsS-RNase* and 22 S-RNases from other species by a Neighbor-Joining (NJ) method. The CsS-RNase had the closest relationship with *Citrus reticulata* (ADA67883.1), and clustered together with *Coffea*, *Antiriihinum*, *Solanum*, *Nicotiana* and *Petunia* (Fig. 3).

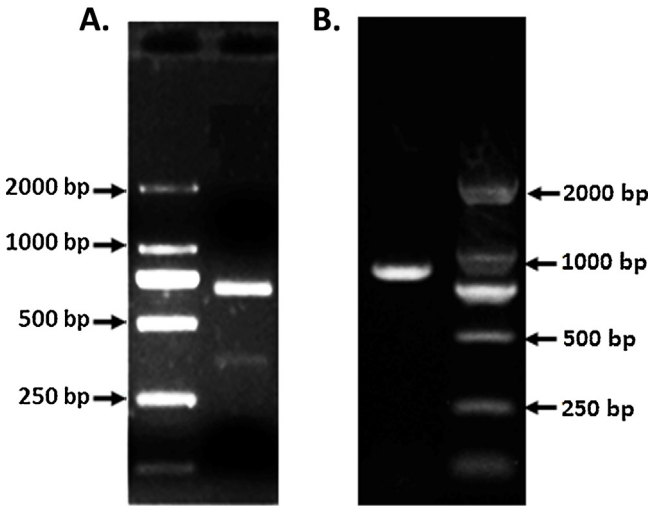

Fig. 1. The target bands of (A) 3′ RACE and (B) full-length cDNA.

3.2. Expression patterns of CsS-RNase

To examine the expression patterns of *Cs-SRNase* in different tissues and self/cross-pollination treatments, sqRT-PCR and qRT-RCR methods were employed. The expression levels of *CsS-RNase* in the styles were 3–259 (FDDB) and 5.6–119 (ZC108) times higher than the petals, ovaries, sepals, pollen grains and leaves (Fig. 4A). This result indicates that Cs-SRNase is mainly expressed in the styles of FDDB and ZC108. Meanwhile, the expression profiles of *CsS-RNase* at different times after self- and cross-pollination in the styles were also detected. The expression levels steadily rose with the pollination time. However, a difference was observed between the two pollination treatments. The expression level of *Cs-SRNase* at 24 h after self-pollinated styles was dramatically higher than in cross-pollinated styles (Fig. 4B–E). Cs-SRNase increased expression in self-pollinated styles earlier than cross-pollinated ones, suggesting that Cs-SRNase plays an important role in self-incompatibility interaction in *C. sinensis*.

3.3. Genotyping of Cs-SRNase in different cultivars

The full-length cDNA of *Cs-SRNases* in 10 cultivars and 1 breeding line were amplified and directly sequenced from both directions. Next, the cDNA sequences were translated into amino acid sequences and aligned using ClustalX2 and MEGA5.2. Eleven polymorphic amino acid residues were obtained. Two cultivars of FDDB and FY6 exhibited allelic polymorphisms. One polymorphic amino acid residue (G↔S) of FDDB is located in a conserved amino acid (CAS) motif, which is characteristic of all RNases T2. Another polymorphic amino acid residue (H↔L) of FY6 occurred in an active

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1 - CGCAAATTAAGAAATAAATAAATACCAATGTGTATGGATATGTGTAGCACATTCCGTCCGACGATGTACATCTTTTGCCT - 80
81 - ATAAATTAAAGCTCTTATGTAATCCAACCATATTGTCTTAATTCTAAAGTCTGAACTCAAATATTGAAGTAAGAGAGAGA - 160
161 - AAAAAACAAGAAAGATGATGTCAAACCCTTCAATCTTGATCAAGCTTTTGGTGGTGCAATGCCTAGCAGTTCTCTGTGTT - 240
1 - K K T R K M M S N P S I L I K L L V V Q C L A V L C - 21
241 - GCTAAGGATTTTGATTTCTTTTACTTTGTTCAACAGTGGCCAGGATCATACTGTGACACAAGGCAAAGTTGCTGCTATCC - 320
22 - A K D F D F F Y F V Q Q W P G S Y C D T R Q S C C Y - 47
321 - AAAGACTGGAAAGCCCGCAGAAGATTTCGGCATTCATGGGCTTTGGCCTAATTATAACGACGGCACTTACCCATCCGGCT - 400
48 - P K T G K P A E D F G I H G L W P N Y N D G T Y P S - 73
401 - GCGATAGCCGCAACTCTTTTGATGACTCTGAGATCTCAGACCTCGCCAGTAGATTGGAAAAAGACTGGCCAACACTAGCA - 480
74 - C D S R N S F D D S E I S D L A S R L E K D W P T L - 99
481 - TGCCCAAGTGGGGATGGGTTGAAGTTCTGGGGACATGAATGGAATAAACATGGGACTTGTGCTGAGTCTGTCTTTGACCA - 560
100 - C P S G D G L K F W G H E W N K H G T C A E S V F D - 125
561 - GCACAGCTACTTCCAAACTGCTCTTGACCTTAAGAACAAAGCCAACCTTCTTCAAGCCCTCACCACTGCAGATATTCGAC - 640
126 - Q H S Y F Q T A L D L K N K A N L L Q A L T T A D I - 151
641 - CAAATGGGAAGTTTTACCACTTAGAGAGCATCAAAGAAGCCATTAGAGAAACTGTTGGAGTCACCCCCTACATTGAGTGC - 720
152 - P N G K F Y H L E S I K E A I R E T V G V T P Y I E - 177
721 - AATGTGGATACATCAGGCAACCACCAGCTCTACCAGGTTTACATGTGCGTCGACTCTTCTGGGTCCAACTTCATCAAATG - 800
178 - N V D T S G N H Q L Y Q V Y M C V D S S G S N F I K - 203
801 - CCCAGTTCTACCCCATAGTCACCCTTGCGGTTCCAAAATCGAATTCCCTTCCTTCTCAGATAACTCAAACTCAAAGAATG - 880
204 - C P V L P H S H P C G S K I E F P S F S D N S N S K - 229
881 - ATGAACTCTGATAACGATATTGTCCTTTTTATTGTTTCATTGATCATCATCATCAGTTGGATTTTAATAATTGCTTGTAG - 960
230 - D E L * - 232
961 - TAACCATCATTCATCGACTATGCGTGGGCGATTTAGTTCTTGTTTTCCTCGGACTTTGCTGCTTTGATCAAGGTCTCATA - 1040
1041 - TATGTAACCACTTTTGATTTGTCAATGAAACAGTGTTTTTATTAAATCAAAAGATCACAATTTTAATCCAAAAAAAAAAA - 1120
1121 - A
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Fig. 2. Full-length cDNA of *CsS-RNase* and its deduced amino acid sequence. The brown background indicates the position of two conserved amino acid motifs of T2 RNase. The red color shows the active sites. Italics indicate the B1 nucleotide binding pocket. Underlines indicate the B2 nucleotide binding pocket.

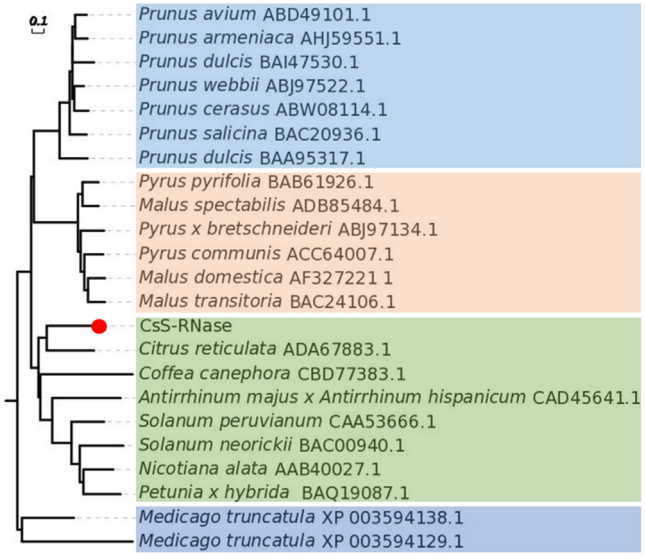

Fig. 3. Phylogenetic analysis of S-RNase amino acid sequences from *C. sinensis* and other species. The red dot shows the position of CsS-RNase.

site of the RNases T2. In addition, LJ43 was consistent with ZC108; 4 cultivars of YS, ZN117, LJCY and ZN139 were consistent with each other; and the breeding line LJ001 was the most different from the other cultivars (Fig. 5).

3.4. *Mapping* Cs-SRNase *on the genetic linkage map*

In order to develop molecular markers for mapping *Cs-SRNase* on the genetic map, a fragment of *Cs-SRNase* was amplified from the genomic DNA of FDDB, LJ43 and BHZ. PCR products from FDDB were purified and sequenced after transformation into competent cells. The sequence was 1129 bp long, including an intron of 645 bp located between the 628 and 629 nucleotide of the cDNA. Next, the fragments from LJ43 and BHZ were directly sequenced from both directions. However, only the backward direction was successfully sequenced with an approximately 550 bp genomic sequence of high quality. After comparing the sequences from the two cultivars, an SNP marker that was heterozygous in LJ43 and homozygous in BHZ was obtained (Fig. 6A). Then, the SNP was converted into a dCAPS marker and detected by restriction enzyme digestion. The target band in LJ43 was successfully digested into two bands, while in BHZ the PCR product was digested into one single band (Fig. 6B). Subsequently, this molecular marker was used to scan the F1 segregating population. As a result, 81 homozygotes and 85 heterozygotes distributed in 166 F1 individuals, with an approximate Mendelian ratio of 1:1 (Fig. 6C). Finally, *Cs-SRNase* was mapped on LG14 of the genetic linkage map (Fig. 6D).

4. Discussion

Self-incompatibility controls the rejection or acceptance of pollen grains, which promotes outcrossing and prevents inbreeding depression in flowering plants (Wheeler et al., 2009). SI is an important agricultural trait for various fruit trees and field crops (Zhang et al., 2015; Liao et al., 2014). However, SI's molecular basis is understood in only a small number of families (Gibbs, 2014). SSI in *Brassicaceae* is controlled by an S-receptor kinase (SRK) and an S-locus cysteine-rich protein (SCR) (Wang et al., 2014). GSI in Rosaceae, Solanaceae and Plantaginaceae is controlled by the S-RNase and the SLF/SFB (Sijacic et al., 2004; McClure, 2006). In this paper, a *CsS-RNase* gene was cloned from *C. sinensis* (Fig. 1). This gene, encoding 238 amino acid residues, belongs to

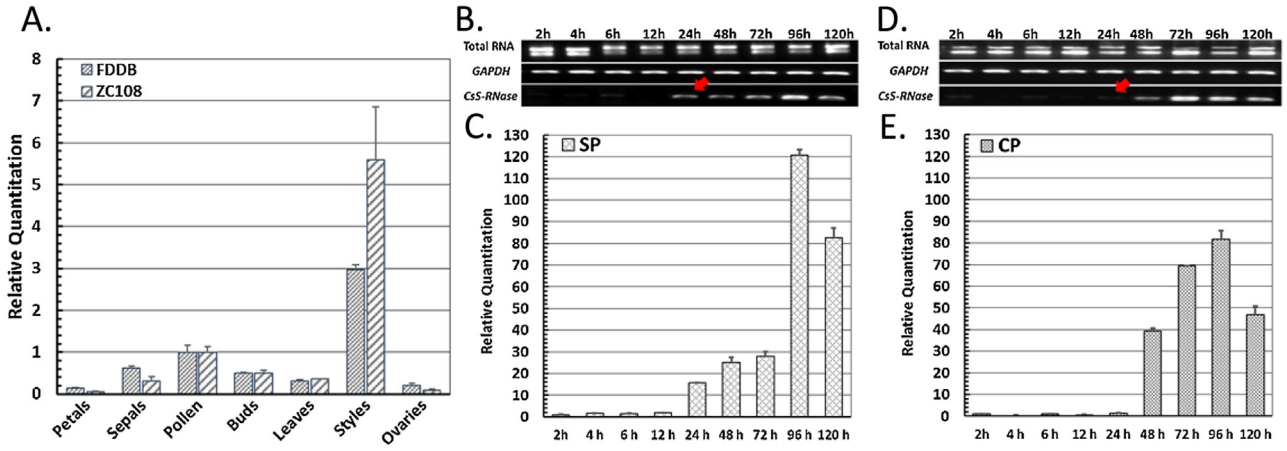

Fig. 4. Expression patterns of *CsS-RNase* in different tissues (A), different times after self-pollination (B and C) and cross-pollination (D and E). The B and D shown the results of sqRT-PCR. The A, C and E exhibited the results of qRT-PCR. The red arrows indicate the different expression levels of *CsS-RNase* in self/cross pollinated styles at 24 h after pollination. Three independent semi-quantitative RT-PCR reactions were performed with similar results and only a representative picture is illustrated here.

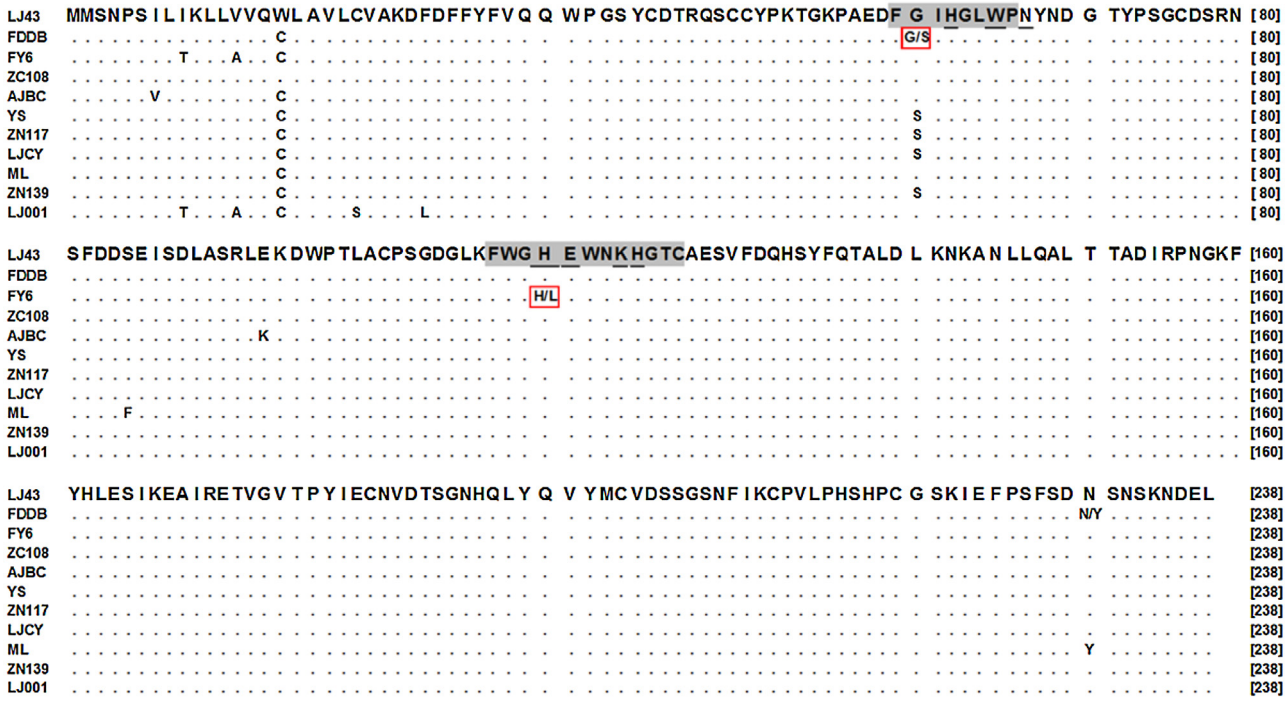

Fig. 5. The amino acid sequences of CsS-RNase in different cultivars. The sequence from LJ43 was used as the reference. The dot indicates amino acids homologous with the reference. The brown background indicates the position of two conserved amino acid motifs of RNase T2 family. The underlines show the active sites. The red boxes indicate the positions of two amino acid changes occurring in a conserved motif (FDDB) and an active site (FY6).

RNase T2 family (Fig. 2). Cluster analysis indicated that the CsS-RNase had the closest relationship with an S-RNase from *Citrus reticulata* (ADA67883.1) (Fig. 3). The latter was cloned from a self-incompatible cultivar 'Wuzishatangju', which was a bud mutant isolated from a self-compatible cultivar of 'Shatangju' (Miao et al., 2011).

S-RNase is a female determinant in the GSI system, which is highly expressed in the styles (Qiao et al., 2004; McClure, 2006; Miao et al., 2011). Here, the expression of *Cs-SRNase* among different tissues was the highest in the styles compared to petals, ovaries, sepals, pollen grains and leaves (Fig. 4A). More importantly, the different expression level of *S-RNase* at different times after pollination is thought to mediate self-sterility. For example, in *Citrus reticulate*, *S-RNase* expression in self-pollinated styles was dramatically higher than cross-pollinated styles at the crucial time of 3 days after pollination. In another example, significantly different expression levels were observed in the pear cultivar 'Nijisseiki' and its self-compatible mutant 'Osa-Nijisseiki' (Sassa et al., 1992). Similarly, different expression levels of S-RNase was observed in the apricot 'Xinshiji' after self- and cross-pollination (Feng et al., 2006). In the present study, *CsS-RNase* was up-regulated in selfed styles earlier than crossed styles (Fig. 4B–E). Therefore, the different expression profiles of *CsS-RNase* between selfed and crossed styles suggests that the *CsS-RNase* might directly mediate the SI reaction in *C. sinensis*.

The flowering stage of *C. sinensis* is through October to November in China. The low temperature, varying weather conditions and long seed developmental periods (nearly 12 months) influence the setting rate and the judgment of compatible or not. Previous studies indicated that determining the genotypes

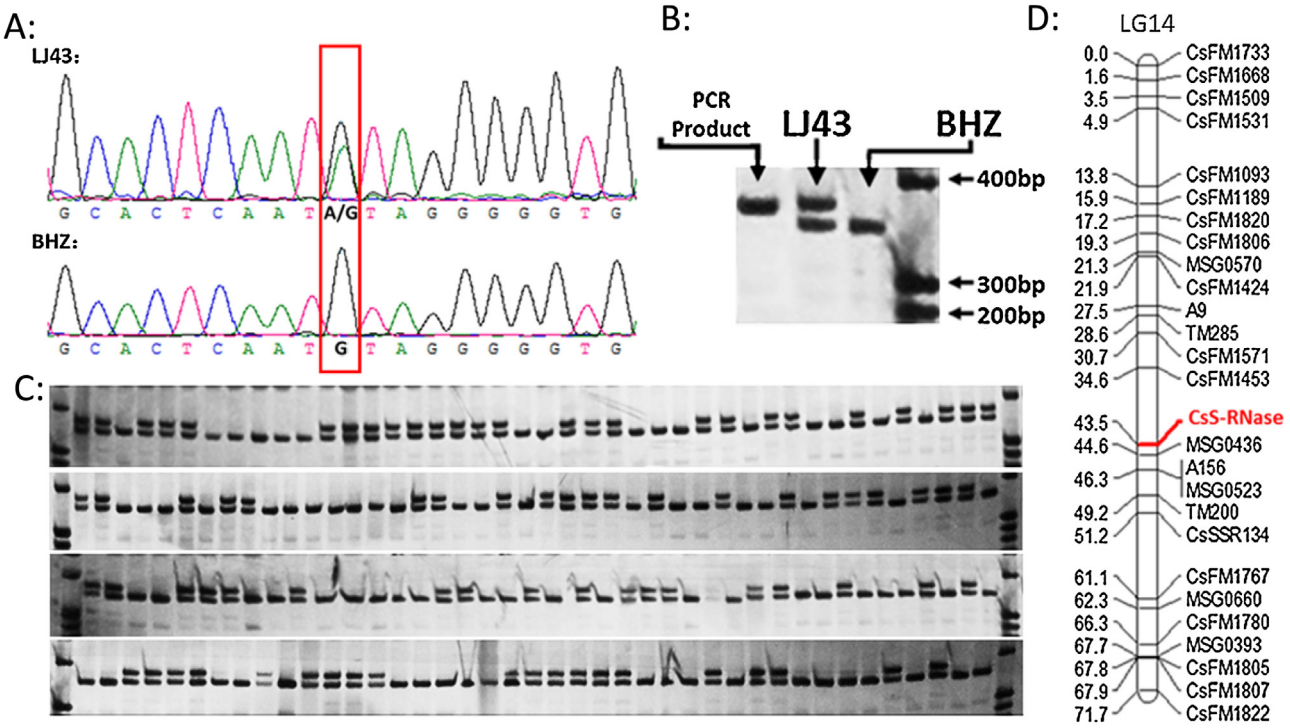

Fig. 6. (A) An SNP marker of *CsS-RNase* was identified in LJ43 and BHZ. (B) The banding pattern of PCR products from 'Longjing43' and 'Baihaozao' digested by *Dra III*. (C) The genotype of *CsS-RNase* in 166 F1 individuals. (D) The *CsS-RNase* was mapped on linkage group 14 (LG14) of the genetic linkage map.

of S-RNase is helpful for parental selection during hybridization breeding (Carrera et al., 2011; Hegedűs et al., 2013). In this study, the CsS-RNase of LJ43 was consistent with ZC108, and the four cultivars YS, ZN117, LJCY and ZN139 were consistent with one another. Therefore, intercrossing these cultivars is not suitable for cross-breeding. In contrast, LJ001 was very different than the other cultivars, which suggests it may be a good crossing parent. Moreover, gene mutations may lead to inactivation of S-RNase, which results in self-compatible mutants. Therefore, detecting genotypes of S-RNase in different cultivars is useful for finding self-compatible individuals (Yaegaki et al., 2001). In the Japanese pear, a self-compatible mutant cultivar 'Osa-Nijisseiki' was caused by a deletion of S^4-*RNase* in the genomic sequence (Sassa et al., 1997). Similarly, a 24 bp insertion occurs in the S6-RNase leading to a self-compatible haplotype in the loquat (Nyska et al., 2013). In addition, 2 self-compatible genotypes in the peach resulted from a premature stop codon mutant and a 4946 bp insertion in *SFB* (Hanada et al., 2014). In this study, *Cs-SRNases* were identified in 10 cultivars and one breeding line of the tea plant. Two polymorphic amino acid residues in a CAS motif (FDDB) and an active site (FY6) might affect the activity of S-RNases in the 2 cultivars (Fig. 5).

GSI is controlled by the S-locus, including pollen and style specificity determinants of *S-RNase* and *SFB/SLF*. The two genes must be tightly linked, otherwise chromosome recombination would disturb the SI trait (Iwano and Takayama, 2012). In *Antirrhinum*, an *AhSLF-*S_2 gene was cloned that was located 9 kb downstream of the S_2-*RNase*. Likewise, in 2 species of cherry (*Prunus cerasus* and *P. avium*), an SFB^6 obtained by inverse PCR was located approximately 380 bp downstream of the S^6-*RNase*. Genetic linkage maps are useful tools for implementing marker-assisted selection (MAS) and quantitative trail loci (QTL) analysis, especially in map-based gene cloning (Somers et al., 2004; Tan et al., 2013). In this study, the *CsS-RNase* was mapped on the genetic linkage map (Fig. 6D), which will helpful for cloning the male determinate *SFB*/SLF gene as well as studying the structure of the *S-locus* in *C. sinensis*.

5. Conclusions

In the present study, a *CsS-RNase* (KU852488) was cloned from *C. sinensis*. This gene was mainly expressed in the styles. The expression of *CsS-RNase* in selfed styles was up-regulated earlier than crossed styles. Therefore, the *CsS-RNase* might be related to SI interaction in *C. sinensis*. The genotypes of this gene were analyzed in 10 cultivars and 1 breeding line of *C. sinensis*, which will useful for parental selection during hybridization breeding. We believe that the cloning and characterization of *CsS-RNase* provides an insight into the SI mechanism in the tea plant, which will further promote breeding research and genetic study in *C. sinensis*.

Acknowledgments

The study was supported by the Modern Agro-Industry Technology Research System (nycytx-23) and the Major Project for New Crop Varieties Breeding of Zhejiang Province (2012C2905-8).

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