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

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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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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-