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Original Research

Molecular mechanisms underlying the participation of Ribonuclease T2 gene into self-incompatibility of *Citrus grandis* var. *Shatianyu* Hort

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Abstract: The *RIB* (ribonuclease T2) of *Citrus grandis* var. *Shatianyu* Hort involved in self-incompatibility (SI) mechanism was identified by prokaryotic expression. RT-qPCR results showed that the expression level of *RIB* in self pollinated stigma is significantly higher than that in cross pollinated stigma. A vector for prokaryotic expression of RIB was constructed after codon-optimization, and the recombinant protein was induced and purified. *In vitro* pollen germination test indicated that the RIB protein markedly inhibited pollen germination and pollen tube growth. The result is helpful for better understanding of the molecular mechanism underlying the SI in *C. grandis*.

Key words: *Citrus grandis* var. *Shatianyu* Hort; Ribonuclease T2; Self-incompatibility.

Introduction

Plant self-incompatibility (SI) means incapable of producing zygotes in hermaphroditic plants after self pollination or cross pollinated by same genotype (1). SI is one of the most known mechanisms that controls fertilization of the hermaphroditic plants, which is a physiological response resulting from pollen-stamen interaction. The nature of SI has been confirmed in more than 3,000 hermaphroditic plants belonging to 250 genus and 74 families (2). SI plays very important role in avoiding inbreeding in sexual reproduction of angiosperm (3). It is also one of the most important driving forces for the rapid evolution of angiosperm, which plays an immeasurable function in the early evolution of angiosperm (4).

According to morphological and genetic classification methods, plant SI is divided into sporophytic self-compatibility (SSI) and gametophytic self-compatibility (GSI) (5). In GSI, the phenotype of pollen is determined by the haploid genotype carried by the gametophyte itself, which has been intensively studied in crucifers, while in SSI, it is determined by the diploid microsporocyte that produces pollen. SSI is more frequently used than GSI, which has been applied in solanaceous, rosaceous, scrophulariaceous and papaveraceous plants. Genetic analysis showed that in most cases the recognition system of SI is controlled by a single locus (S-locus) of a multiple allele. Because there was a strong negative selection at S-locus, several multiple alleles were appearing at one S-locus as a result (6). And there was a sequence diversity among different multiple alleles (7). At the molecular level, it was showed that S-locus has at least two multiple alleles that control SI, one encodes male determinant and the other encodes female determinant (8, 9). These two determinants associate closely in terms of genetics. They link or interchange as a genetic unit and pass to the next generation. If a plant producing pollen or stamen has the same S-locus, and this resulting pollen falls to the same pistil, pollen growth will be inhibited, called SI.

Lewis reported the first protein associated with specific S-locus allele in a GSI plant, *Oenothera organesis* (10), using an immunological method. In 1980s, the GSI protein was isolated, identified and sequenced, and its distribution as well as property was studied. The proteins linked to SI were purified and identified from the stigma of *Prunus avium, Lycopersicon peruvianum, S. tuberosum* and *S.chacoense* in tandem. These proteins are alkaline glycoprotein with ca. 25-32 kD molecular weight and 8.6-10.0 PI (11-13). They are mainly distributed in the intercellular substance of stigma's transfer cells and the highest concentration was found at the upper 1/3 part of the stigma. This is the site where pistil recognizes and inhibits incompatible pollen (14). *In vitro* experiments showed that purified S protein and crude extract of stigma both could inhibit pollen tube growth (15). In 1986, Anderson et al. Cloned S_2-cDNA, a gene of S-glycoprotein related to GSI, and in 1989, they isolated the cDNA of S-protein at loci S3 and S6 (16). According to sequence alignment, proteins encoded by these genes have very high homology to fungal RNaseRH and RNaseTH (17). These proteins have nuclease activity and can degrade pollen RNA, leading to the occurrence of SI (18). To date, about 100 S-RNase genes have been isolated from solanaceous, rosaceous, scrophulariaceous, and Chinese cabbage plants (19-21).

Shatianyu (*C. grandis* var. *Shatianyu* Hort) belongs to Citrus, Rutaceae. It is an excellent citrus variety with large plantation area. However, due to its innate SI, it is

time consuming and laborious for production because reasonable arrangement of pollinating plants or artificial pollination are necessary. In practice, a number of citrus varieties are capable of parthenocarpy. If these varieties have SI, no pollinating trees will be necessary during production and seedless fruits can be obtained. This is one of the objectives searched by citrus breeders. Therefore, it is of theoretical and practical significance for an investigation of the innate SI mechanism in citrus plants. Previous research showed that Shatianyu belongs to GSI (22). Domestic and abroad scholars have conducted a series of study concerning the cell biology and molecular basis of the GSI in Shatianyu. The type of SI and the site where pollen tube growth of Shatianyu was inhibited were identified; S-glycoprotein in stigma of SI Shatianyu was isolated and purified by bidirectional electrophoresis and affinity chromatography; the S-glycoprotein was proven having similar biochemical activity, physiochemical property and nuclease activity with S-RNase from other species; results of double immunodiffusion of pollen protein extract and S1-RNase suggested there were components in the pollen (pollen tube) of Shatianyu responsive to SI-RNase (23-27). Although plant SI has been studied in some citrus plants, the mechanisms of SI in the citrus, rutaceae, remains unclear (28).

In order to investigate the SI mechanism in Shatianyu, we have previously analyzed the difference of gene expression profile of self and cross pollinated stigma of Shatianyu by using digital gene expression profiling technology (DGE). We annotated and found an mRNA (Unigene2441_All) that has the same conversed CAS domain with Ribonuclease T2 (RNase T2). In the present study, we constructed a vector for prokaryotic expression of this sequence (gene), induced and purified the recombinant protein and tested the effects of this protein on pollen germination and growth by *in vitro* pollen tube germination test. Results of the study will deepen our understanding to the molecular mechanism underlying the SI in Shatianyu.

Materials and Methods

Material preparation

Stigma of Shatianyu was collected from Chaotian village, Lingchuan County, Guangxi Autonomous Region, China. During the flowering period of Shatianyu, stigmas were treated as follows. (1) For self crossing, stigmas were smeared with self pollen and then enclosed by waterproof paper. (2) For out crossing, the stamen was removed at first, stigma was pollinated by pollen of *Citrus grandis*, and then enclosed by waterproof paper. (3) Un-pollinated materials (stigma) were fresh un-pollinated flowers. The un-pollinated, 1d, 2d and 3d pollinated stigmas after self crossing or out crossing were collected. The stigma materials were flash frozen in liquid nitrogen and stored at −80°C until RNA extraction.

RNA extraction

One, two and three days after Shatianyu were cross pollinated or artificially self pollinated, the pollinated and un-pollinated stigmas of Shatianyu were collected. Total RNA of these pollinated and un-pollinated stigmas of Shatianyu was extracted with TRIzol reagent. The total RNA was treated by RNase-free DNAseI to remove genomic DNA. Eukaryotic mRNA was enriched by magnetic beads with Oligo (dT).

Real-time quantitative PCR analysis (RT-qPCR)

(1) Reverse transcription: 0.5μl RNase inhibitor (50U/μl), 2μl random primer (50pM/μl) and 2μl RNA were added to a centrifugal tube and the volume was adjusted to 10.5μl by DEPC water. After vortexed, the centrifugal tube was heated in 65°Cwater bath for 5 min, cooled down at room temperature for 10 min, centrifugated at 5000 r/min for 5 seconds, and then 0.5μl RNase inhibitor (50U/μl), 4μl of 5×buffer, 2μl dNTP MIX (10mM/each), 2μl DT, and 1μl AMV (200U/ul) was added and total volume was adjusted to 20ul by adding ddH_2O. The resulting mixture was vortexes and then warmed in 40°C water bath for one hour, heated at 90°C for 5-10min, cooled down in ice bath for 5min and then centrifuged at 5000 r/min for five seconds. (2) Fluorescence quantitative PCR amplification: sequence of Unigene2441_All was selected from results of sequencing. Primers were designed by Primer Express Software v2.0 (ABI Company). The primer sequences were (from 5' to 3'), RIB-F: GCCTAATTACAACGACGGCT, and RIB-R: GGCCAGTTCTTTAGCATGCT. The internal reference gene was *GAPDH*. The reaction mixture for Real-time PCR includes 8μl of 2×PCR MIX (QIAGEN), 0.2 μl each forward and reverse Primers (50pM/ul), 1μl Template (cDNA) and 7.6μl ddH_2O. The condition for RT-PCR was 95°C for 2min, 94°C for 10s, 58°C for 10s and 72°C for 40s. The entire process was 40 cycles. Each sample was replicated three times at a 384-well plant with ABI Vii 7 PCR instrument. Ct value was set according to the modified value. The internal reference gene for qRT-PCR was GAPDH and results were quantified by $2^{-\Delta\Delta Ct}$.

Construction of vector for RIB prokaryotic expression

The full length sequence of Unigene2441_All

```
GGAAACTCTTACAAGGCTTCTTCTATACATAGATACATTCAAAACCATTGTCGATCTCCC
TCCCAAAAATTAATATTGATTTTACTATTGGCACTTTGAGAATTATGGAGTGCAAGCGCC
                                                M  E  C  K  R
AGTTTTCAATAATCTTGATCAAACTTTTCTTCATTCAGTATCTATCAGTTCTTTGTGCTG
Q  F  S  I  I  L  I  K  L  F  F  I  Q  Y  L  S  V  L  C  A
CCCGGAACTTCGATTTCTTCTACTTTGTCCTGCAGTGGCCAGGATCATACTGTGATACAG
A  R  N  F  D  F  F  Y  F  V  L  Q  W  P  G  S  Y  C  D  T
CGAAGAGTTGCTGCTATCCAACGACTGGAAAGCCAGCAGCAGATTTCGGGATTCATGGAC
A  K  S  C  C  Y  P  T  T  G  K  P  A  A  D  F  G  I  H  G
TCTGGCCTAATTACAACGACGGCTCCTATCCATCCAACTGTGACCCCAATGCCCCTTTCG
L  W  P  N  Y  N  D  G  S  Y  P  S  N  C  D  P  N  A  P  F
ATCAATCCCAGATATCAGACCTGCGAAGCAGCATGCTAAAGAACTGGCCAACACTGGCTT
D  Q  S  Q  I  S  D  L  R  S  S  M  L  K  N  W  P  T  L  A
GCCCAAGCGGGAATGGCATAACATTTTGGTCCCATGAATGGGAGAAACACGGCACTTGCT
C  P  S  G  N  G  I  T  F  W  S  H  E  W  E  K  H  G  T  C
CTGAGTCTGTGCTTAACCAACATCAGTACTTTCAAACAGCTCTTAACCTGAAAAATCAAA
S  E  S  V  L  N  Q  H  Q  Y  F  Q  T  A  L  N  L  K  N  Q
TCAATCTCCTCCAAGCTCTCAGAACCGCAGGAATAGTGCCTGACGGGAGTTCGTACAGCT
I  N  L  L  Q  A  L  R  T  A  G  I  V  P  D  G  S  S  Y  S
TGGAAAGCATCAAGGATGCGATCAAAGAAGCAAGTGGGTTCAGTCCATGGATAGAGTGCA
L  E  S  I  K  D  A  I  K  E  A  S  G  F  S  P  W  I  E  C
ATGTTGATGAATCAGGCAACAGCCAGCTTTATCAGATTTACTTGTGTGTCGACACTTCTG
N  V  D  E  S  G  N  S  Q  L  Y  Q  I  Y  L  C  V  D  T  S
CCTCTAACTTCATCAACTGCCCCGTCTTTCCCAACGGCAAAAAATGTGGATCCCAGATTG
A  S  N  F  I  N  C  P  V  F  P  N  G  K  K  C  G  S  Q  I
AGTTTCCTCCGTTTTAGTGATCAAGGGATCAATAATCAACGTGTGTGATTAATTTCTTCA
E  F  P  P  F  *
CCATGTTAAGCTGATTCAATTTCAGTCTTTTTTATTATTGATCAATCTCATATTGTTCTA
CCAAACTAGTTGCTCAAGTAATTATTTCCTAGCCAGTAGCCGTTAAGGTGGTAATGGCTT
CTTTTATTTGATGTTCAAATTGAATTGCTTGTCAAGTTATTGTCTTCTCAAACTAGATAT
AAAGTTTTATGGATGGTCCTACGCTCCA
```

Figure 1. Gene sequence of RNase-like storage protein and its putative amino acid sequence.

contains 1048 bp (GenBank access No.: KR363153) and named as RIB. Based on the analysis of NCBI ORF Finder and DNAman software (NCBI), the sequence includes one ORF containing 693bp. It encodes 230 amino acids (Figure 1). The molecular weight (MW) is 25.82 kDa and pI is 5.30.

Amino acid sequence was analyzed by the Conserved Domain Architecture Retrieval Tool (NCBI). The protein was found to have a same conserved CAS domain with the Ribonuclease T2 (RNase T2). In order to confirm the function of protein encoded by this sequence, codons of this sequence were optimized. Signal peptide sequence of 29 amino acids at 5' end was artificially synthesized (Figure 2) and ligated to pUC57 plasmid. The target gene was then cleaved by NdeI/HindIII and then cloned to pET43.1a plasmid to construct a eukaryotic expression vector.

Expression, purification and functional test of RIB protein

(1) Induced expression of recombinant protein: plasmid containing RIB protein gene was transformed to BL21 (DE3) competent cells of *E. coli*, smeared evenly on LB medium containing Amp and then incubated overnight in a 37℃ oven. Monoclone was inoculated on LM medium containing Amp. When OD600 was 0.5-0.8, 0.375 mM IPTG was added to the test tube containing culture solution and induced at 15℃ for three hours. 2 ml of *E. coli* solution was centrifuged and *E.coli* strain was obtained. 200 µl of PBS solution containing 1% of Triton X-100 and 3 mmol.L^{-1} of PMSF was added to the *E.coli* strain and ultrasonicated. 80µl of supernatant was collected and to which 20µl of 5×SDS-PAGE was added. The mixture was then denatured and expression of target protein was examined by SDS-PAGE. (2) Purification of recombinant protein: one ml of *E.coli* stock was inoculated with 200 ml of liquid LB culture medium containing 100ug/ml of Amp, and then induced overnight at 15℃. The resulting *E.coli* strain was ultrasonicated. The supernatant was the crude extract containing histidine-tagged fusion protein. The fusion protein was absorbed by Nickel column and eluted by imidazole elution. The recombinant protein was purified by molecular sieve and condensed by desalination. The concentration of recombinant protein was determined by Bradford serum determination kit. The protein was then subjected to SDS-PAGE and confirmed by Western Blot.

Analysis of recombinant protein activity by pollen germination

The medium for pollen cultivation contains 12% sucrose, 0.02% $MgSO_4$, 0.01% KNO_3, 0.03% $Ca(NO_3)_2$, 0.01% boric acid and pH value of the culture medium is 5.6. Different concentrations of RIB protein (0, 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 µg/µl) were added to the culture medium. Defrosted pollens were spread evenly in the liquid medium and incubated in a 25℃ oven in dark. Ten hours later, the percentage of pollen germination and length of pollen tube was determined with a microscope. Five visual fields were observed for every concentration and there were at least 20 pollens in every visual field. Length of pollen tube > diameter of pollen grain was considered as germination. The length of 20 pollen tubes was measured in three replicates.

```
Unigene2441 Protein | Length=230 MW=25818.84 PI=5.3
   1 MECKRQFSII LIKLFFIQVL SVLCAARNFD FFYFVLQWPG SYCDTAKSCC YPTTGKPAAD
  61 FGIHGLWPNY NDGSYPSNCD PNAPFDQSQI SDLRSSMLKN WPTLACPSGN GITFWSHEWE
 121 KHGTCSESVL NQHQYFQTAL NLKNQINLLQ ALRTAGIVPD GSSYSLESIK DAIKEASGFS
 181 PWIECNVDES GNSQLYQIYL CVDTSASNFI NCPVFPNGKK CGSQIEFPPF
```

Figure 2. Amino acid sequence encoded by Unigene2441_All.

```
Unigene2441 Protein | Length=208 MW=23290.8 Predicted pI=5.94
1    MHHHHHHDFF YFVLQWPGSY CDTAKSCCYP TTGKPAADFG IHGLWPNYND GSYPSNCDPN
61   APFDQSQISD LRSSMLKNWP TLACPSGNGI TFWSHEWEKH GTCSESVLNQ HQYFQTALNL
121  KNQINLLQAL RTAGIVPDGS SYSLESIKDA IKEASGFSPW IECNVDESGN SQLYQIYLCV
181  DTSASNFINC PVFPNGKKCG SQIEFPPF
```

Figure 3. Amino acid sequence encoded by optimized codons (easy for eukaryotic expression).

Results

Isolation and structural analysis of RIB Gene

Through alignment and analysis, we found one mRNA (Unigene2441_All) that has nuclease activity. By homogenous sequence amplification, we obtained the full-length sequence of this mRAN (GenBank accession No.: KR363153). The full length of this mRNA is 1048bp. Homology of amino acid sequence coded by this mRNA was 99% as compared to that of *Citrus sinensis*, XP_006469367 and *Citrus clementina*, XP_006438697. Based on an analysis by ORF Finder and the bioinformatics software DNAman (NCBI), RIB contains one ORF that encodes 230 amino acids including a signal peptide containing 15 amino acids at N terminal. The length of this ORF is 693bp. Molecular weight of RIB protein is 25.82 kDa and the pI is 5.30. This protein has a same conserved CAS domain with the Ribonuclease T2 (RNase T2) protein (Figure 4).

Expression of RIB gene after self and cross pollination by real-time quantitative PCR analysis (RT-qPCR)

After self and cross pollinated, the expression level of *RIB* gene in the self and cross pollinated stigmas of Shatianyu differed considerably (Figure 5). The expression of *RIB* gene in un-pollinated stigma was the lowest. The expression of *RIB* gene in 1-3d cross pollinated stigmas was slightly increased but did not differ markedly. The expression of *RIB* gene in 2d self pollinated stigmas increased markedly, but decreased slightly at 3d. However, the expression level was higher than that in the 3d cross pollinated stigmas.

Optimized RIB gene sequence

In order to ensure efficient expression of *RIB* gene in prokaryotic cells, we optimized the sequence of *RIB* gene without changing its amino acids. The optimized sequences of nuclear acid and amino acid sequence of RIB gene were presented in figure 6 and 7, respectively. In addition, we predicted the signal peptide encoded by this protein by SignaIP software (http://www.cbs.dtu.

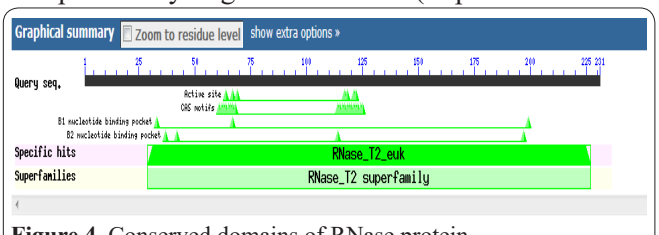

Figure 4. Conserved domains of RNase protein.

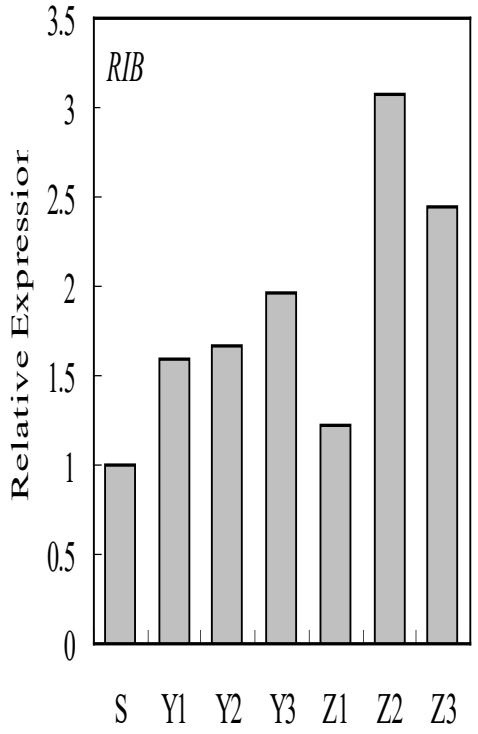

Figure 5. Expression of *RIB* gene after self and cross pollination**.** S = un-pollinated stigma; Y1 = stigma after one day cross pollination; Y2 = stigma after two day cross pollination; Y3 = stigma after three days cross pollination; Z1 = stigma after one day self pollination; Z2 = stigma after two day self pollination; Z3 = stigma after three day self pollination.

dk/services/SignaIP/). We found one signal peptide at N terminal of this protein. Therefore, we removed this 15-amino acid signal peptide at the N terminal. Molecular weight of target protein was 23.29 kDa.

Expression and Purification of RIB Protein

Expression of RIB gene was induced in *E. coli* BL21 (DE3) containing recombinant plasmid. Results of SDS-PAGE showed that the *E. coli* BL21 (DE3) containing recombinant plasmid has produced a unique protein band after induced by 0.375mM IPTG. Molecular weight of this protein was 23kDa, which was consistent as expected. The *E. coli* BL21 (DE3) without induction by 0.375mM IPTG failed to produce this band (Figure 8).

Scaled up cultivation and purification of RIB protein: *E.coli* BL21(DE3) containing recombinant plasmid was amplified to 2L and induced overnight at 15℃. The resulting *E.coli* strain was harvested, ultrasonicated, purified by Ni2+-NTA (QIAGEN) and eluted by 10-500 mmol·L^{-1} of imidazole solution (10, 20, 40, 60, 80, 100, 150, 200, 250, 500). The optimal concentration was 150 mmol·L^{-1} (Figure 9).

Molecular weight identification of RIB protein: molecular weight of RIB protein was analyzed by Western Blot. The primary antibody was anti-His-tagged monoclonal antibody of mouse. The secondary antibody was horseradish peroxides-coupled goat anti-mouse IgG. RIB protein was dyed by DAB. There was a specific band at 23kDa (Figure 10), which was consistent with the result of SDS-PAGE.

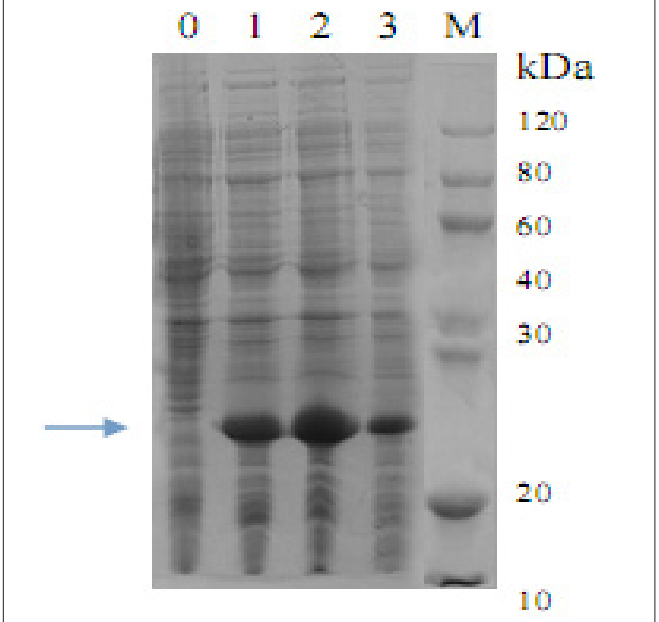

Figure 8. Expression of RIB protein in BL21 (DE3) by SDS-PAGE. Lane M: SDS-PAGE Protein marker; Lane 0: control; Lane 1: induced overnight at 15℃; Lane 2: induced overnight at 28℃; Lane 3: induced overnight at 37℃.

Effects of RIB Protein on Pollen Germination and Pollen Tube Growth

The pollen germinated in the liquid culture medium after 2h cultivation. 10h later, the length of pollen tube was almost 3/4 of the total length and 24h later the pollen tube ceased to grow. Germinated pollen grains were counted to calculate germination rate (a standard for pollen germination is that the length of pollen tube is

```
1     CATATGCACC ATCACCATCA CCATGACTTC TTTTACTTCG TTCTGCAGTG GCCGGGTTCA
61    TATTGCGATA CCGCGAAAAG CTGCTGTTAT CCGACCACCG GTAAACCGGC AGCAGATTTT
121   GGTATCCACG GTCTGTGGCC GAATTACAAC GACGGTAGCT ATCCGAGCAA TTGCGATCCG
181   AACGCACCGT TTGATCAGTC TCAGATCAGC GATCTGCGCT CTAGCATGCT GAAAAATTGG
241   CCGACCCTGG CTTGTCCGTC TGGTAACGGC ATTACCTTTT GGTCTCACGA GTGGGAAAAA
301   CACGGTACCT GTAGCGAAAG CGTCCTGAAT CAGCATCAGT ACTTTCAGAC CGCGCTGAAC
361   CTGAAAAACC AGATCAACCT GCTGCAAGCA CTGCGTACCG CAGGTATTGT TCCGGACGGT
421   AGCAGCTATA GCCTGGAAAG CATTAAAGAC GCGATCAAAG AAGCGAGCGG CTTTTCTCCG
481   TGGATTGAGT GCAACGTTGA CGAAAGCGGT AACAGCCAGC TGTATCAGAT CTACCTGTGC
541   GTTGATACCT CTGCGAGCAA CTTTATCAAC TGCCCGGTCT TTCCGAACGG CAAAAAATGC
601   GGTAGCCAGA TTGAATTTCC GCCGTTC
TAA TGAAAGCTT
```

Figure 6. Sequence of optimized *RIB* gene.

```
1     MHHHHHHDFF YFVLQNPGSY CDTAKSCCYP TTGKPAADFG IHGLWPNYND GSYPSNCDPN
61    APFDQSQISD LRSSMLKNWP TLACPSGNGI TFWSHEWEKH GTCSESVLNQ HQYFQTALNL
121   KNQINLLQAL RTAGIVPDGS SYSLESIKDA IKEASGFSPW IECNVDESGN SQLYQIYLCV
181   DTSASNFINC PVFPNGKKCG SQIEFPPF
```

Figure 7. Amino acid sequence of optimized RIB.

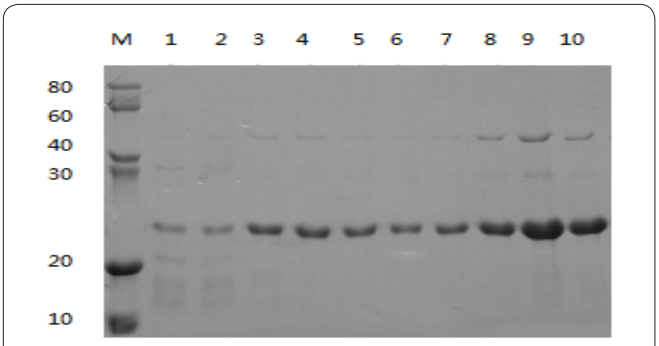

Figure 9. SDD-PAGE test for RIB protein after purification by Ni-NTA affinity chromatography. Lane M: SDS-PAGE Protein marker; Lane 1-10: results of elution by different concentrations of imidazole (0, 20, 40, 60, 80, 100, 150, 200, 250, 500 mmol.L^{-1}).

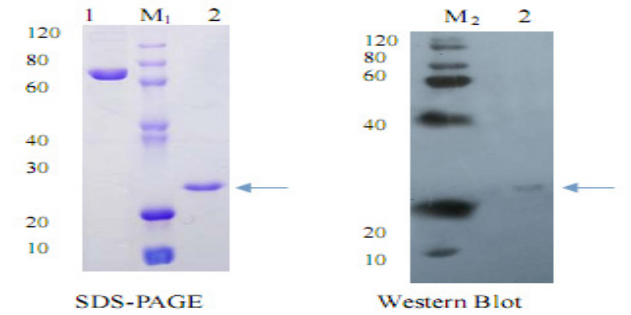

Figure 10. Identification of recombinant protein by SDS-PAGE and Western Blot. Lane 1: BSA (2.0ug); Lane 2: RIB protein; M1: SDS-PAGE Marker; M2: Western Blot Marker; (Note: Anti-His antibody was used in Western Blot).

larger than the diameter of pollen). In the *in vitro* pollen germination test of Shatianyu, RIB protein markedly inhibited pollen germination and pollen tube growth, which was the same as those for pearl and tobacco (29, 30).

Discussion

In 1980s, Clarke lab isolated a glycoprotein binding to specific haplotype from the stigma of a solanaceous plant, *Nicotiana alata* (16). The first *S*-gene participating into solanaceae SI was identified by N terminal sequencing and subsequent cDNA cloning. About 100 S-RNase genes were isolated from a number of plants as yet. These S-RNase genes encoded about 250 amino acids (31, 32). Based on protein sequence of these amino acids in S-RNase, these protein sequences contained two highly variable regions and several conserved regions (33). The highly variable regions are assumed to associate with the separation and recognition process of S-RNase gene, and most of them contain hydrophilic

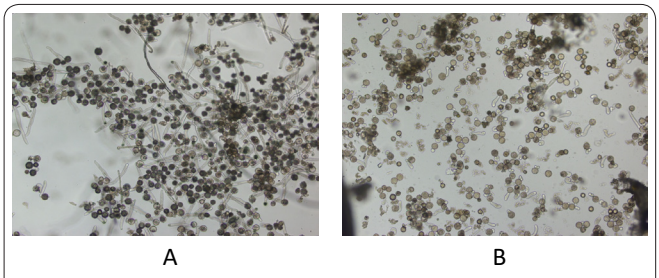

Figure 11. Condition of pollen tube germination. A : CK; B : 1.5 ug/ul RIB protein; cultivated in dark at 25°C and calculated after 10h cultivation.

S-protein domain. The conserved region is assumed to be a component of S-RNase and to maintain S-RNase activity. The amino acids encoded by the mRNA obtained in the present study have more than 96% homology to the S-RNase of *Citrus maxima* and sweet orange, suggesting that our mRNA was the *RNase-like* storage protein gene. By analyzing the functional domain of protein encoded by this gene, we found that this protein has a same conserved CAS domain as the Ribonuclease T2 protein. The RNase T2 usually has conserved sites (CAS), (CAS I and CAS II), and both have similar amino acid sequence, F/WTL/IHGLWP and FWXHEWXKHGTC. When RNase-like storage protein gene and the conserved sites of RNase T2 were aligned, the conserved amino acid residuals at locus 1 (Phenylalanine, F), locus 2 (Tryptophan, W) and locus 3 (Threonine, T) in CAS I region were replaced by tyrosine (Y), phenylalanine (F) and valine (V), respectively, whereas the conserved site in CAS II region was completely same.

The authors ever studied the gene expression at locus S, but most of our studies were exclusively focused on the tissue-specific expression. Chai et al (34) found the expression of F-box protein at locus S in style, petal and anther of Zigui shatian pummelo ten days after flowering, but no expression was detected in leaf and ovary. The expression of another S-like RNase gene *CgSL2* in Zigui shatian pummel was different. The highest expression of *CgSL2* was observed in stamen, followed by petal, and the expression was lowest in stem (35). In the present study, the expression level of *RIB* gene at locus S was significantly higher in self pollinated stigma than in un-pollinated and cross pollinated stigmas. This supplies another proof to the participation of genes at locus S into citrus SI. Similar result was not reported in previous studies.

Exogenous protein usually shows low expression level, poor solubility, false folding and aggregation in prokaryotic expression system. Optimization of base sequence of target gene is a frequently used solution in practice depending on host (36, 37). We optimized the nuclear acid sequence of RIB gene without changing its amino acid sequence, and got pure RIB protein after induction in *E.coli*. Molecular weight of RIB protein was 23kDa, which as consistent as expected. At present, almost all S-proteins that control SI in stigma are exclusively glycoproteins with 20-40 kDa MW and 5.0-8.0 pI (34, 38). In the present study, the MW of S-glycoprotein purified by us in stigma was 23.29kDa and its pI was

Table 1. Effects of RIB protein on pollen germination and pollen tube growth of Shatianyu.

Protein concentration ($ug.uL^{-1}$)	Mean length of pollen tube (μm)	Pollen germination rate (%)	Significance (0.05)
0	152.7764±13.1618	70.89± 6.23	a
0.3	48.2458±7.5049	42.21±7.22	b
0.6	47.9013±4.3567	41.33±6.23	b
0.9	42.7835±3.5900	40.78±6.94	b
1.2	40.8929±5.7024	36.55±4.56	b
1.5	40.7482±3.3972	36.34±3.35	b
1.8	40.3214±2.3063	36.21±5.67	b

Data were analyzed by SPSS 19 software. 0 means control. There is significant different between the control group and treatment group. No significant difference was observed among different treatment groups.

5.58. Our results were in agreement with the biochemical properties of previously reported S-protein. Our finding will supply favorable basis to the study of protein function in the future.

S-protein plays very important role in SI. It is thus of significant to reveal its mechanism and to determine the biological function of S-protein. S-protein probably has a universal function. In corn, S-glycoprotein inhibited the growth of homogenous pollen tube *in vitro*, but not affected pollen germination (39). Similar results were observed in *Petunia hybrid*, *Nicotiana alata*, Papaver and other SI plants (40, 41). In *in vivo* studies, the inhibition of pollen tube growth in self pollinated plants occurred when the pollen tube grew to a certain length, at a specific stage or some time after pollination (42). It was found in *Nicotiana glauca* that S-glycoprotein inhibited pollen growth in a dosage-response manner. S-RNase is a secretary glycoprotein. Most of S-RNase is present in the intercellular gap of passage tissue in stigma and expressed specifically in pistil. During SI process, the highest S-RNase concentration was found at the upper 1/3 part of the stigma where pollen growth was strongly inhibited (16). We found in the present study that RIB protein markedly inhibited pollen tube growth and pollen germination of Shatianyu. This inhibitory effect occurred at 0.3 $\mu g.\mu L^{-1}$, but not increased with increasing treatment concentration. Therefore, it is assumed that trace amount of RIB can inhibit pollen germination and pollen tube growth, thereby leading to SI. It was already reported that the expression level of S-RNase glycoprotein is closely related to flower development. SI occurs only when S-RNase level gets to a threshold. During the flower bud period, S-RNase level in stigma can't satisfy with the requirement for pollen tube growth inhibition. So the expression level of S-mRNA and S-RNase glycoprotein was very low, thereby leading to SI. Our results also confirmed this conclusion. In addition, in living Shatianyu plants, pollen tube growth was inhibited and ceased when it grew to the upper 1/2 of the stigma and the ovary can't be fertilized as a result (43). Based our results, RIB protein is probably secreted by conducting tissues in stigma, and plenty of RIB protein is expressed and transported to sticky medium of intercellular gap 3d after the stigma was self pollinated; germinated pollen tube grows in the sticky medium; self pollinated pollen tube (wall) probably contains a special material (receptor) that can specifically recognize RIB. Stigma RIB protein can enter the pollen tube and functions as nuclease, degrades rRNA inside, thereby inhibiting protein synthesis in pollen tube and consequently pollen tube growth.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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