1 **SLFL Genes Participate in the Ubiquitination and Degradation of S-RNase in**

2 **Self-compatible Chinese Peach**

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15 **Title**

16 ***SLFL* Genes Participate in the Ubiquitination and Degradation of S-RNase in**
17 **Self-Compatible Chinese Peach**

18

19 **Abstract**

20 The gametophytic self-incompatibility (SI) mediated by S-RNase of Rosaceae,
21 Solanaceae and Plantaginaceae, is controlled by two tightly linked genes located at
22 highly polymorphic S-locus: the S-RNase for pistil specificity and the F-box gene
23 (SFB/SLF) for pollen specificity, respectively. The F-box gene of peach (*Prunus*
24 *persica*) is S haplotype-specific F-box (*SFB*). In this study, we selected 37
25 representative varieties according to the evolution route of peach and identified their S
26 genotypes. We cloned pollen determinant genes mutant *PperSFB1m*, *PperSFB2m*,
27 *PperSFB4m* and normal *PperSFB2*, and style determinant genes *S1-RNase*, *S2-RNase*,
28 *S2m-RNase* and *S4-RNase*. Mutant *PperSFBs* were translated terminated prematurely
29 because of fragment insertion. Yeast two-hybrid showed that mutant *PperSFBs* and
30 normal *PperSFB2* interacted with all S-RNases. Normal *PperSFB2* was divided into
31 four parts: box, box-V1, V1-V2 and HVa-HVb. Protein interaction analyses showed
32 that the box portion did not interact with S-RNases, both of the box-V1 and V1-V2
33 had interactions with S-RNases, while the hypervariable region of *PperSFB2*
34 HVa-HVb only interacted with *S2-RNase*. Bioinformatics analysis of peach genome
35 revealed that there were other F-box genes located at S-locus, and of which three
36 F-box genes were specifically expressed in pollen, namely *PperSLFL1*, *PperSLFL2*

37 and *PperSLFL3*, respectively. Phylogenetic analysis showed that PperSFBs and
 38 PperSLFLs were classified into two different clades. Yeast two-hybrid analysis
 39 revealed that as with PperSFBs, the three F-box proteins interacted with PperSSK1.
 40 Yeast two-hybrid and BiFC showed that PperSLFLs interacted with S-RNases with no
 41 allelic specificity. In vitro ubiquitination assay showed that PperSLFLs could tag
 42 ubiquitin molecules to PperS-RNases. In all, the above results suggest that three
 43 *PperSLFLs* are the appropriate candidates for the ‘general inhibitor’, which would
 44 inactivate the S-RNases in pollen tubes, and the role of three PperSLFL proteins is
 45 redundant, as S-RNase repressors involved in the self-incompatibility of peach.

46

47 **Key words:** Chinese peach, self-compatibility, S-locus F-box like gene (*SLFL*),
 48 S-RNase, ubiquitination, SCF complex

49

50 **Introduction**

51 Self-incompatibility (GSI) is the most widely distributed breeding system that
52 allows the pistil to reject genetically related pollen and promotes out-crossing in
53 flowering plants (de Nettancourt, 2001). Many species in Solanaceae, Rosaceae, and
54 Plantaginaceae exhibit S-RNase-based GSI which is controlled by the complex
55 S-locus which contains at least two genes, one gene is pistil-part, a highly
56 polymorphic *S* gene encoding extracellular ribonuclease called S-RNase, and one is
57 pollen-part specific *S* gene, which is tightly linked to the S-RNase. The tightly linked
58 genetic unit of the pistil *S* allele (S-RNase) and pollen *S* allele is called *S* haplotype.
59 The pollen *S* genes of the S-RNase-based GSI of the above three families are F-box
60 genes called *SLF/SFB* (Entani et al., 2003; Lai et al., 2002; Sijacic et al., 2004;
61 Ushijima et al., 2003; Yamane et al., 2003). The pollen *S* gene was called *SLF* (*S*
62 locus F-box) in Solanaceae and Plantaginaceae and *SFB* (*S* haplotype-specific F-box)
63 in *Prunus* (Sassa et al., 2010; Tao and Iezzoni, 2010; Meng et al., 2011). S-RNase is
64 secreted into style transmitting intercellular space, and non-selectively taken up into
65 cytoplasm of compatible and incompatible pollen tubes elongating in style tissues,
66 and pollen tube elongation is arrested in incompatible crosses, probably because of
67 cytotoxic effects of self S-RNase (Luu et al., 2000; Goldraij et al., 2006; McClure et
68 al., 2011; Boivin et al., 2014). The predominant role of F-box proteins is
69 ubiquitination of proteins, thus possibly *SLF/SFB* acts as a subunit to generally
70 constitute the SCF complex, an E3 ubiquitin ligase, discriminates between self and
71 non-self S-RNase, and mediate the ubiquitination of non-self S-RNases for

72 degradation by the 26S proteasome (Lechner et al., 2006; Franklin-Tong, 2008; Hua
73 et al., 2008). In this process, how F-box proteins discriminate self and non-self
74 S-RNases in pollen tubes is unknown.

75 A single S-locus F-box gene is known as *SFB* identified in *Prunus* (Entani et al.,
76 2003; Ushijima et al., 2003; Sonneveld et al., 2005), while multiply *F-box* genes
77 located at the S-locus have been cloned in subtribe Maloideae designated as *F-box*
78 *brothers* (Sassa et al., 2007; Kubo et al., 2010; Kakui et al., 2011). In subfamily
79 Maloideae (e.g., apple and pear) of Rosaceae, polyploidization breaks SI in pollen but
80 does not affect the pistil (de Nettancourt, 2001). The pistil of ‘Fertility’ (2x) could
81 accept pollen from autotetraploid (4x), but ‘Fertility’ (2x) pollen was rejected by the
82 pistil of autotetraploid (4x) (Crane and Lewis, 1942). Genetic analyses reveal that the
83 breakdown of SI can be explained by ‘competition’ between different S alleles in
84 pollen. But in *Prunus* (subfamily Prunoideae), tetraploidy is not always associated
85 with SC. Sour cherry (*Prunus cerasus*) is a naturally occurring tetraploid species and
86 includes both SI and SC plants (Lansari and Iezzoni, 1990). Genetic analysis of SI
87 sour cherry suggested that heteroallelic diploid pollen tubes are rejected by pistils
88 with cognate S haplotypes (Hauck et al., 2006). Hauck et al. (2006) proposed that the
89 breakdown of SI in tetraploid sour cherry is caused by the accumulation of
90 non-functional S haplotypes and not by competitive interaction in heteroallelic pollen.
91 In Japanese pear, S^{4sm} pollen lacking $SFBB1-S^4$ are rejected by compatible S^1 pistils
92 but accepted by S^3 and S^5 pistils (Okada et al., 2004 ; Okada et al., 2008). On the other
93 hand, loss-of-function of $SFBB1-S^5$ had no effect on SI phenotype. Genetic analysis

reveals that S⁵ pollen is normally accepted by S¹, S³ and S⁴ pistils (Sassa et al., 2011). The previous fruit set analyses shows that S⁵ pollen is normally compatible with S² and S⁹ pistils and incompatible with S⁵ pistils (Kajiura et al., 1967; 1969; 1974). On the contrary, in *Prunus*, a truncated SFB protein or lacked the SFB gene can confer pollen-part self-compatibility (SC) (Ushijima et al., 2004; Sonneveld et al., 2005; Hauck et al., 2006; Tsukamoto et al., 2006). These findings suggest that S-RNase-based GSI seems to consist of two types in which the mode of action of pollen S is different, a ‘self recognition by a single factor’ system and a ‘non-self recognition by multiple factors’ system (Kakui et al., 2011), and the S-RNase-based GSI of *Prunus* represents ‘self recognition by a single factor’, in which the cytotoxic effect of non-self S-RNases in pollen tubes is inactivated by a ‘general inhibitor’ while the self S-RNase is specifically protected by a ‘blocker’ molecule and degrades RNA of self-pollen to arrest tube growth (Luu et al., 2001; Sonneveld et al., 2005). Although the ‘general inhibitor’ is a hypothetical protein and had been considered to be SLFLs in *Prunus avium* (Matsumoto and Tao, 2016), and in peach, whether the ‘general inhibitor’ is SLFLs as with in *Prunus avium* needs to be studied.

Skp1, Cullin1 (Cul1), Rbx1 and F-box proteins together constitute the SCF complex, E3 ubiquitin ligases. The E3 ubiquitin ligase can make substrate proteins polyubiquitination to degrade by the 26s proteasome system. In the SCF complex, the F-box protein determines substrate specifically, Skp1 serves as an adaptor to connect the variable F-box protein and Cul1 protein, Cul1 forms a core catalytic scaffold with Rbx1, and Rbx1 can bind to E2 and catalyzes the transfer of ubiquitin chains from E2

116 to the substrate protein to make ubiquitination of substrate proteins (Wu et al., 2000;
117 Zheng et al., 2002; Deshaies and Joazeiro, 2009). SLF/SFBB is shown to be a
118 compoment of the SCF complex to detoxified non-self S-RNases. In petunia, SLF was
119 showed to form the SCF complex with Skp1-like and Cull1-p in pollen(Zhao et al.,
120 2010; Entani et al., 2014; Liu et al., 2014), and in Maloideae, SFBB was also shown
121 to form SCF complex which targeted selectively S-RNase and polyubiquitinated it in
122 vitro (Yuan et al., 2014).

123 In our study, we selected 37 representative species according to the evolution
124 route of peach, and identified their S genotypes. Through yeast two-hybrid and BiFC
125 analysis, we found that PperSFB2 distinguished self S2-RNase from non-self
126 S-RNases by the C-terminal hypervariable region. According to the genome wide
127 analysis, we cloned three *SLFL* genes in the *S*-locus on chromosome 6, and did some
128 experiments and analysis to determine whether the function of the *SLFL* genes is the
129 same as that of *Prunus avium*. Our results showed that PperSLFL proteins were in
130 different clade compared with PperSFB proteins, and could participate in
131 self-incompatibility of peach as a subunit of SCF complex.

132

133 **Materials and Methods**

134 **Plant Material**

135 37 peach varieties (Supplemental Table 1) were selected from the Zhengzhou
136 Fruit Research Institute, Chinese Academy of Agricultural Sciences, Henan Province,
137 China. Peach organs/tissue samples (leaves, styles and pollen) were collected, frozen

138 in liquid nitrogen and stored at -80°C Ultra-low temperature refrigerator for later use.

139

140 **DNA and RNA Extraction**

141 Peach genomic DNA was isolated from young leaves using the CTAB method
 142 (Li et al., 2009), and incubated with RNase I (Invitrogen, CA, USA) at 37°C for 2
 143 hours to remove RNA. Total RNA samples were isolated from leaves, styles and
 144 pollen using a modified CTAB method (Li et al., 2009) and treated with DNase I
 145 (Invitrogen, CA, USA) to remove DNA contamination. RNA was used as template to
 146 synthesize first-strand cDNA using the SuperScript reverse transcriptase (Invitrogen,
 147 CA, USA) and Oligo-dT primers (According to manufacturer's instructions,
 148 Invitrogen, CA, USA).

149

150 **PCR for S Genotype Analysis**

151 Peach genomic DNA was used as templates for PCR with the primers listed in
 152 Supplemental Table 2. The primers were designed according to the length of the
 153 second intron of the *S*-RNases. The different *S*-RNases and *S* genotypes of peach
 154 varieties could be distinguished depending on the size of the amplified fragments.

155

156 **Cloning of *PperS-RNases*, *PperSFBs*, *PperSLFLs*, *PperSSK1*, *PperCUL1* and** 157 ***PperRbx1***

158 Pollen cDNA was used as template to clone, *PperSFBs*, *PperSLFLs*, *PperSSK1*,
 159 *PperCUL1* and *PperRbx1* and style cDNA was used as template to clone

160 *PperS-RNases* with the gene specific primers listed in Supplemental Table S2. The
 161 PCR products were purified and individually ligated to the pMD19T-simple vector
 162 (TaKaRa).The constructed vectors were transformed into *E. coli* competent DH5α
 163 cells (Transgene biotech, Beijing, China). Each gene selected 3 positive clones for
 164 sequencing.

165

166 Tissue-specific expression analysis

167 cDNA samples synthesized from total RNA from leaves, styles and pollen of the
 168 37 peach varieties included in this study were used as templates to analyze
 169 tissue-specific expression of *PperS-RNases*, *PperSFBs* and *PperSLFLs*. Gene specific
 170 primers were designed and listed in Supplemental Table S2, and an actin gene was
 171 used as an internal control for constitutive expression with the following thermal
 172 cycling condition: a denaturation step at 94°C for 5 min followed by 30 cycles of
 173 95°C for 30 s, 60°C for 30 s. 72°C for 30, and then 72°C for 10 mins.

174

175 Construction of the phylogenetic tree of F-box, CUL1 and SSK1

176 64 CDSs of S-locus F-box genes from *Malus domestica*, *Pyrus pyrifolia*, *Pyrus*
 177 *bretschneideri*, *Prunus avium*, *Prunus dulcis*, *Prunus mume*, *Prunus salicina*, *Prunus*
 178 *armenica* were used to construct phylogenetic trees with the f-box genes specifically
 179 expressed in pollen cloned from 37 peach varieties in this study. The deduced amino
 180 acid sequences of Skp1-like proteins and cullin-like proteins from *Arabidopsis*
 181 *thaliana*, *Antirrhinum hispanicum*, *Prunus tenella*, *Petunia integrifolia*, *Pyrus*

182 *bretchneideri*, *Malus domestica*, *Prunus avium*, *Prunus persica*. *Petunia integrifolia*,
183 *Vitis vinifera*, *Nicotiana tabacum*, *Prunus tomentosa* and *Prunus mume* were aligned
184 by CLUSTALW. Based on the alignment, phylogenetic trees were constructed by the
185 neighbor-joining method (Saitou and Nei, 1987) using MEGA 6.0 program with the
186 neighbor-joining method and the bootstrap test replicated 1000 times. The confidence
187 values were shown on the branches.

188

189 **Yeast Two-hybrid (Y2H) Analysis**

190 Yeast transformation and activity of β -galactosidase assays were performed
191 following the manufacturer's instructions (Clontech, CA, USA). The partial CDSs of
192 *PperS-RNases* removed signal peptide and the full-length CDSs of *PperSSK1* and
193 *PperCUL1* were cloned into pGBKT7 vector (Clontech), whereas the full-length
194 CDSs of *PperSFBs*, *PperSLFLs*, *PperSSK1* and *PperRbx1* were cloned into pGADT7
195 vector (Clontech).

196 In order to find out the reason that *PperSFB* differentiates between self S-RNase
197 and non-self S-RNases, Normal *PperSFB2* was divided into four parts: box, box-V1,
198 V1-V2 and HVa-HVb and cloned them into pGADT7 vectors. Yeast two-hybrid assay
199 was performed to observe the interactions between different portions of *PperSFB2*
200 and all *PperS-RNases* in this study.

201 For the Y2H assay, AH109 cells containing both AD and BD plasmids were
202 grown on SD/-Leu/-Trp medium for 3 d at 30°C. Ten independent clones for each
203 combination were streaked on SD/-adenine/-His/-Leu/-Trp medium and grown for 3-4

204 d at 30 °C. Then use X- a-gal (TaKaRa Bio) to dye the clones to determine if there
205 were interactions between the combinations. For quantitative measurements,
206 β -galactosidase activity was determined using o-nitrophenyl- β -D-galactopyranoside
207 (Sigma Aldrich) as a substrate according to Hao J. (2009) described.

208

209 **Bimolecular fluorescence complementation (BiFC) analysis**

210 The pCambia1300 vector was used to construct BiFC vectors, which contained
211 the N- or C-terminal of yellow fluorescence protein (YFP) fragments (YFPN and
212 YFPC), respectively. The full-length CDSs of *SLFLs* without stop codon were cloned
213 into pCambia1300-YFPN vectors, whereas the partial CDSs of S-RNases without stop
214 codon removed signal peptide were cloned into pCambia1300-YFPC. All the
215 construct vectors were transformed into *Agrobacterium tumefaciens* GV3101 and
216 co-infiltrated into *Nicotiana Benthamiana* leaves. Fluorescence was observed in
217 epidermal cell layers after 5 days by Olympus BX61 fluorescent microscope
218 (Olympus FluoView FV1000).

219 The box, box-V1, V1-V2 and HVa-HVb frames without the stop codon were
220 amplified and cloned into the pCambia1300-YFPN vectors. The recombinant
221 plasmids containing the *box-YFPN*, *box-V1-YFPN*, *V1-V2-YFPN* or *HVa-HVb-YFPN*
222 fusion gene and *PperS1-RNase*, *PperS2-RNase*, *PperS2m-RNase* or *PperS4-RNase*
223 removed signal peptide fusion gene and the control plasmid with *YFPN* and *YFPC*
224 were co-transformed into maize (*Zea mays* Linn.Sp.) protoplasts respectively
225 according to Ren et al. (2011). GFP fluorescence was observed by Olympus BX61

226 fluorescent microscope (Olympus FluoView FV1000).The primers used were listed in
227 (Supplemental Table 2).

228

229 **Purification of Peptide Tagged Recombinant Proteins**

230 His-tagged proteins were purified as previously described (Meng et al., 2014).

231 The mature peptide of the S-RNases without signal peptide were cloned into the
232 pEASY-E1 vector (From TransGen Biotech Company) and transformed into the *E.*

233 *coli* strain BL21 plysS (DE3) (From TransGen Biotech Company). The cells were

234 inoculated into LB medium containing 100μg/ml ampicillin and incubated for about

235 3h at 37 °C in a shaker at 200 rpm. Once the cell suspension OD₆₀₀ reached about 0.5,

236 isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into medium with final

237 concentration of 0.2 mM to induce protein expression. The cell suspension was

238 incubated for 10-12 h at 16°C in the shaker at 180rpm. His-tagged fusion proteins

239 were purified using Ni-NTA His Binding resin (Novagen, USA) as previously

240 described (according to the manufacturer's instructions of Ni-NTA His Bind Resins,

241 Novagen). The full-length coding sequences of pollen-expressed *PperSFB1m*,

242 *PperSFB2m*, *PperSFB2*, *PperSFB4m* *PperSLFL1*, *PperSLFL2* and *PperSLFL3* were

243 cloned into pMAL-c5x vector, which is designed to generate maltose-binding protein

244 (MBP) fusion proteins. Similarly, the *PperCUL1*, *PperSSK1* and *PperRbx1* were

245 cloned into pGEX4T-1 vector, which is designed to produce glutathione S-transferase

246 (GST) fusion proteins. All the GST-fusion proteins and MBP-fusion proteins were

247 purified using glutathione resin and maltose, as previously described (Yuan et al.,

248 2014).

249

250 ***In Vitro* Ubiquitination Analysis of S-RNase**

251 *In vitro* ubiquitination assays were performed as previously described (Yang et
252 al., 2009; Yuan et al., 2014). The reaction mixture containing 50 mM Tris (pH 7.4), 10
253 mM MgCl₂, 2 mM dithiothreitol (DTT), 5 mM HEPES, 2 mM adenosine triphosphate
254 (ATP), 0.05% Triton X-100, 10 mM creatine phosphate, 1 unit of phosphokinase, 10
255 µg ubiquitin, 50 nM E1 (UBA6, *Petunia hybrida*), 1 mM PMSF, 850 nM E2 (UBH6,
256 *P. hybrida*), and aliquots of the recombinant proteins GST-PpSSK1, GST-CUL1,
257 GST-Rbx1 and any MBP-SLFL at 30 °C for 2 h. Mixtures were immunoblotted using
258 an anti-S-RNase antibody (From Beijing ComWin Biotech Company).

259

260 **Results**

261 **Identification of *PperS-RNase* and *PperSFB* alleles in 37 Chinese peach varieties**

262 For this study, we collected 37 Chinese peach varieties which represent local
263 cultivars in 18 provinces/municipalities in China (Supplemental Table 1). These are
264 thought to represent the evolutionary paths from the origin in central China (Tibet,
265 Yunnan and Guizhou provinces) to the northwest of China (Shanxi province), then to
266 the southwest of China and finally to the coastal and Xinjiang provinces (Cao et al.,
267 2014) (Supplemental Fig. S1). Only four *S*-haplotypes *S*1, *S*2, *S*2m and *S*4, were
268 detected from 36 peach varieties except Guang He Tao (Supplemental Fig. S2)
269 (Supplemental Table 1), and they had previously been reported (Tao et al. 2007). The

270 *S* genotype of 18 varieties, including Da Hong Pao, were *S2S2* genotype and 9
271 varieties, including Hunchun Tao, were *S1S2* genotype, while 3 varieties were of the
272 *S2S4* genotype (Feicheng Bai Li 10, Feicheng Bai Li 17 and Feicheng Hong Li 6)
273 (Supplemental Table 1). The *S2-RNase* in 6 varieties was observed to contain a
274 nucleotide substitution (G→A), which results in the conversion of the sixth conserved
275 cysteine residue to a tyrosine in the *Prunus* C5 domain (Fig. 1A). This was named as
276 *S2m-RNase*. *S2-RNase* and *S2m-RNase* were also identified from the original species
277 Guang He Tao, which indicating that the mutation of *S2-RNase* had occurred before
278 the formation of Chinese peach cultivars. Interestingly, we found that two *S-RNases*
279 cloned from Guang He Tao lacked two introns (Fig. 1A). The 4 *S-RNases* were only
280 expressed in pistil (Fig. 1D).

281 The mutated *PperSFBs* cloned from all varieties in this study were the same as
282 previously reported mutations, but we found that the sequence of inserted 155 bp
283 fragment in *PperSFB1m* was the same as the sequence of 155 bp fragment upstream
284 of the insertion point, and the sequence of 5bp insertion in *PperSFB2m* was also the
285 same as the 5 bases upstream of the insertion point. The sequences of 351 bp at both
286 ends of the inserted 4949 bp fragment in *PperSFB4m* were also the same (Fig 1B).
287 The mutant repeat sequences of *PperSFB4m* had previously been reported (Toshio et
288 al., 2014). In addition, except the mutated *SFB2m*, a canonical *SFB2* gene was cloned
289 from Guang He Tao, indicating that peach mutations occurred prior to evolution and
290 the canonical *SFB2* was eliminated during the selection process. The expression of
291 *SFBs* was pollen-specific (Fig. 1D).

292

293 **Cloning and expression analysis of *PperSLFL* and SCF complex (*PperCUL1*,**
 294 ***PperSSK1* and *PperRbx1* genes)**

295 According to the sequences of *S-locus F-box-like*s (*SLFLs*) in peach genome
 296 (Genome Database for Rosaceae, <http://www.rosaceae.org/>), the primers were
 297 designed to clone the six *SLFL* genes. Finally, only three *SLFL* genes were cloned
 298 from pollen cDNA and their expression was pollen-specific (Fig. 1D). We named the
 299 three F-box genes as *PperSLFL1*, *PperSLFL2* and *PperSLFL3*, respectively.
 300 *PperSLFL1* located at about 47kb downstream of *PperS2-RNase* and the translation
 301 direction was opposite to that of *S2-RNase*; *PperSLFL2* located at about 26kb
 302 downstream of *S2-RNase* and *PperSLFL3* located at about 1.3kb upstream of
 303 *S2-RNase*, and the translation directions of the both F-box genes were the same as that
 304 of *S2-RNase*. The three *PperSLFL* genes did not have introns (Fig. 1C). The
 305 *PperSLFL1* and *PperSLFL2* showed pollen-specific expression, similar to the
 306 *PperSFB* genes. Despite the fact that *PperSLFL3* transcripts were detected in both
 307 leaves and styles, its expression was the most in pollen (Fig. 1D). The identity of the
 308 predicted amino acid sequences of *PperSLFL1*, *PperSLFL2* and *PperSLFL3* was
 309 52.45%, while the alignment of the predicted amino acid sequences of *PperSLFL1*
 310 with *PperSLFL2* and *PperSLFL3*, respectively, the identity was 31.33% and 31.72%,
 311 respectively. The 3 *PperSLFL* genes showed low sequence identity with each other
 312 and with *PperSFB*. All the *PperSLFL* proteins contained the basic F-box domain and
 313 the FBA domain (Fig. 2). The phylogenetic tree analysis showed the three *PperSLFL*

genes of peach clustered together with other *Prunus SLFL* genes, which was reported previously (Tao et al., 2008). The phylogenetic tree had two large lineages; the *Prunus SFB* genes did not cluster together with the pollen S genes of *Pyrus* and *Malus* and diverged into a separate lineage. *Pyrus* and *Malus SFBs* and *Prunus SLFL* genes clustered together and diverged into two secondary lineages. *Prunus SLFL* genes were more closely related to *Pyrus* and *Malus SFB* genes than *Prunus SFB* genes (Supplemental Fig. S3A).

The SSK1 proteins with two conserved domains of apple, pear and petunia were used in a BLAST search for the proteins predicted in the peach predict protein database (<http://www.rosaceae.org/>). The candidate *SSK1* gene (*PperSSK1*) was cloned with specific primers (Supplemental Table 2). The full-length coding sequence (CDS) of *PperSSK1* was amplified from ‘Hunchun Tao’ (*SIS2*) pollen and was subsequently identified in the other 36 peach varieties. The canonical Skp1 comprises 150 to 200 amino acid residues and contains a Skp1-POZ domain at the N terminus and a Skp1 domain at the C terminus. The deduced amino acid sequence of *PperSSK1* comprised 177 residues and contained the Skp1-POZ and the Skp1 domain. In the phylogenetic tree, *PperSSK1* clustered into a lineage with *PtSSK1* and *PavSSK1* (Supplemental Fig. S3B). In addition, the other two subunits of the SCF complex, *PperCUL1* and *PperRbx1*, were cloned with pollen cDNA of ‘Hunchun Tao’ (*SIS2*) as template. The candidate *PperCUL1* gene encoded the protein containing 744 amino acid residues and phylogenetic analysis showed that it clustered together with *MdCUL1* and *PavCULB* which have been shown to be a component of the SCF

336 complex (Tao et al., 2016; Yuan et al., 2014) (Supplemental Fig. S3C). PperRbx1
337 protein contained 117 amino acid residues, and had an H2 loop figure domain at the
338 C-terminus which is necessary for ubiquitin ligase activity.

339

340 **The Interactions between S-RNases and S-locus F-boxes**

341 Yeast two-hybrid analysis was performed to detect the interactions of PperSFBs
342 with S-RNases. *PAI* (ppa011133m) (Aguilar et al., 2015), also a T2-RNase of *Prunus*
343 *persica*, was cloned in this study. Because of no signal peptide, the full-length coding
344 sequence (CDS) of *PAI* was cloned into pGBKT7 to detect the interactions of PA1
345 with S-locus F-boxes. The results showed that the mutated PperSFB and normal
346 PperSFB2 interacted with all the S-RNases, and these interactions displayed no allelic
347 specificity. There was no interaction between PA1 and PperSFB (Supplemental Fig.
348 S4A). Furthermore, the β -galactosidase reporter gene activity was detected and it
349 showed that the intensity of interactions between these combinations was not high and
350 the intensity of interaction between normal PperSFB2 and S2-RNase was slightly
351 higher than that of other combinations (Supplemental Fig. S4B). Because of the
352 insertion of the fragments, the proteins encoded by *PperSFB1m*, *PperSFB2m* and
353 *PperSFB4m* genes were terminated prematurely and the domains at C-terminus was
354 lost in varying degrees. In order to explore the effect of each part of the SFB on
355 S-RNase, we divided the normal *PperSFB2* gene into four parts: box, box-V1, V1-V2
356 and HVa-HVb (Supplemental Fig. S4C). Using yeast two-hybrid and bimolecular
357 fluorescence complementation (BiFC) analysis, we found that the box region did not

358 interact with all of four S-RNases, whereas box-V1 and V1-V2 portions of PperSFB2
 359 physically interacted with all four S-RNases, and the interactions displayed no allelic
 360 specificity. From the yeast coloring time stained by X- α -gal and fluorescence intensity,
 361 it could be seen that the interaction intensity between each combinations was not high.
 362 Interestingly, the HVa-HVb of PperSFB2 only interacted with S2-RNase but not other
 363 S-RNases, indicating a potential role in S-RNase-SFB specific recognition
 364 (Supplemental Fig. S5).

365 The expression of *PperSLFL1-3* had pollen specificity and contained F-box
 366 domains, which led to a problem that whether they might play roles in
 367 self-incompatibility of peach. First, we detected the interactions of PperSLFL1-3 with
 368 S-RNases. The Y2H and BiFC analysis were performed using various fusion
 369 expression vectors containing *S-RNases* or *PperSLFL1-3*, respectively. The Y2H
 370 assay showed that S-RNases interacted with PperSLFL1-3 with no allelic specificity,
 371 respectively (Fig. 3A). The β -galactosidase report gene activity suggested that the
 372 intensity of interaction between PperSLFL1 and the four S-RNases was slightly
 373 higher than other various combinations (Fig. 3E). PA1 did not interact with any
 374 PperSLFL proteins (Fig. 3A). To confirm that results, bimolecular fluorescence
 375 complementation (BiFC) experiment was performed in *Nicotiana tabacum* leaves.
 376 The BiFC results also indicated that PperSLFL1-3 interacted with the four *S-RNases*
 377 with no allelic specificity (Fig. 4).

378

379 **Interaction analysis of S-locus F-boxes, PperSSK1, PperCUL1 and PperRbx1**

380 The yeast two-hybrid analysis was performed to investigate the interaction
381 between PperSSK1 and PperSFBs/PperSLFLs, and the interaction between
382 PperCUL1 and PperSSK1/PperRbx1. The results indicated that PperSSK1 interacted
383 with PperSFB2, PperSFB1m, PperSFB2m and PperSFB4m (Fig. 3C), and the activity
384 of β -galactosidase confirmed the intensity of the interaction was high (Fig. 3D). Both
385 PperSSK1 and PperRbx1 interacted with PperCUL1, and the activity of
386 β -galactosidase analysis quantitatively demonstrated the interaction between them
387 (Fig. 3B).

388 We also examined the interactions between PperSSK1 and three other
389 pollen-expressed F-box proteins, PperSLFL1–3, to explore the hypothesis that
390 PperSLFL proteins might play a role in self-incompatibility of peach. The yeast
391 two-hybrid analysis was conducted using the full-length CDSs of these 3 S-locus
392 F-box protein genes. All the three F-box proteins showed interaction with PperSSK1
393 (Fig. 3C). The activity of β -galactosidase report gene quantitatively demonstrated the
394 interaction between them, and the interaction between PperSSK1 and PperSLFL1 and
395 PperSLFL2 was stronger than that of between PperSSK1 and PperSLFL3 (Fig.3D).

396

397 **A SCF complex containing PperSLFL1-3 ubiquitinates S-RNases in vitro**

398 In order to test whether S-RNases could be ubiquitinated by SCF^{SLFL} in vitro,
399 commercial His-UBA6 was used as the ubiquitin-activating (E1) and His-UBH6 was
400 used as ubiquitin-conjugating enzyme (E2). Purified MBP-PperSLFL1-3,
401 GST-PperSSK1, GST-PperRbx1 and GST-PperCUL1 were used as E3. Anti-MBP

antibody was used to detect MBP-PperSFB proteins and MBP-PperSLFL proteins by Western blot analysis, anti-S-RNase antibody was used to detect S-RNase proteins and ubiquitinated S-RNases and anti-GST antibody was used to detect GST-tagged proteins. Purified His-S-RNase proteins, MBP-SFB proteins and MBP-SLFL proteins were detected a single band (Fig. 5). Distinct immunoreactive bands with higher molecular masses (between 34 KDa and 130 KDa) were detected above the predicted His-S-RNase (26 KDa) bands, and no band was detected in the negative control reactions without His-S-RNase proteins (Fig. 6). The results indicated that PperSLFL proteins could ubiquitinate S-RNase of peach.

Discussion

In this study, we selected 37 Chinese peach varieties from 18 areas of China, including the ancestral species (Guang He Tao), wild species (Qing Si, Huo Lian Jin Dan, Qing Mao Zi Bai Hua, Bai Nian He, Zhang Bai 5 and Long 1-2-4) and some common local varieties. After identifying the S-genotypes of all the peach varieties in this study (Supplemental Table 1), four previously described S haplotypes were identified S1, S2, S2m and S4. S2 was the most frequent S-haplotype in the tested Chinese peach varieties (occurred in 33 varieties), followed by S1 (in 10 varieties), S2m (in 7 varieties) and S4 (only in 3 varieties). All four S haplotypes, S1 S2, S2m, S4, found in this study had the same mutant versions as that reported previously (Tao et al., 2007; 2010). The mutated *S2m-RNase* and *SFB2m* genes, canonical *SFB2* gene and *S2-RNase* existed in Guang He Tao indicating that these mutations occurred

424 before the formation of Chinese peach cultivars. In the mountains of Tibet, peach
425 might be propagated by seeds generally obtained from genotypes with high
426 productivity. Because of the special natural environment, self-compatibility generally
427 led to more reliable fruit set, which made the probability of survival in natural
428 selection increased, but the genetic diversity in the S-locus was declined. We
429 proposed that under selection pressure for SC, pollen part mutants might
430 preferentially be selected compared to pistil part mutants because there were many
431 pollen grains and the pollen genotype in a large extent affected the SI phenotype in
432 GSI system. That is why all the peach S haplotypes characterized in this study are
433 pollen part mutant S haplotypes and the S2-allele accumulates the most.

434 After analyzing the sequences of mutant pollen *S* genes, we found that the 155bp
435 fragment inserted in *PperSFB1m* was duplicated from the 155bp region upstream of
436 the insertion point, and the 5 bp fragment inserted in *PperSFB2m* was the same with
437 the 5 bp upstream of the insertion point. The sequence of 351 bp at both ends of the
438 inserted 4949bp fragment in *PperSFB4m* was also the same that had been reported
439 (Fig. 1B) (Tao et al., 2010). That kind of mutant might be due to an error in
440 homologous recombination, or a retro-transposition. As we know, gene duplication is
441 a ubiquitous biological phenomenon, an important driving force for the diversification
442 of genomic and genetic systems, and plays a very important role in the evolution of
443 biological processes. This repetition in peach might have a significant for the study of
444 its evolution.

445 In the S-RNase-based GSI system, non-self S-RNases in pollen tube are

446 detoxified. It is hypothesized that detoxification of non-self S-RNases in pollen is
 447 mediated by the SCF complex. S-RNases are degraded by SCF^{SLF/SFBB} mediating in
 448 other plant species with the S-RNase-based GSI system (Tao and Iezzoni, 2010;
 449 Iwano and Takayama, 2012; Yuan et al., 2014). In this study, three pollen-expressed
 450 F-box genes (Fig. 1D and Fig. 2), named as PperSLFL1-3, located at the S locus of
 451 peach (Fig 1C). Phylogenetic and evolutionary analysis indicated that PperSLFL1-3
 452 clustered with SLFLs of other *Prunus* species on the same evolutionary branch, and
 453 the evolution relationship between SLFLs and SFBBs of apple and pear was closer
 454 than the evolution relationship between SLFLs and SFBs (Supplemental Fig. S3),
 455 which was the same as Tao did (Tao et al., 2008). We speculated that the *Prunus* SI
 456 recognition mechanism might have some differences compared to the mechanism in
 457 the Maloideae. Researchers speculated that *Prunus* self-incompatibility mechanism
 458 was ‘self recognition by a single factor’ system (Sonneveld et al., 2005). In the ‘self
 459 recognition by a single factor’ system, the cytotoxic effect of non-self S-RNases is
 460 thought to be inactivated by an unidentified ‘general inhibitor’ (GI) (Sonneveld et al.,
 461 2005). The single factor we speculated was SFB, because the peach became
 462 self-compatibility from self-incompatibility due to SFB mutant. SFB2 had a role in
 463 self/non-self recognition, the variable region interacted with self/non-self S-RNase,
 464 and the hypervariable region interacted with self-S-RNase (Supplemental Fig. S5).
 465 We suspected that SFB specifically recognizes self-S-RNase to leave self-S-RNase
 466 active, leading to the arrest of self pollen tube growth.

467 The F-box protein would be the good candidate for the GI. In sweet cherry,

468 S-RNases were recognized by PavSLFL2 (Matsumoto, 2016). In this study,
 469 PperSLFL1-3 interacted with all the S-RNases with no allelic specificity (Fig. 3A),
 470 and phylogenic analysis demonstrated that SLFLs including PperSLFL1-3 were
 471 classified into the same clade as SFBB of the Maloideae. Since it is plausible that SI
 472 or the S locus of Prunus and the Maloideae share the same origin (Igic and Kohn,
 473 2001; Steinbachs and Holsinger, 2002), we suspect that *SLFLs* are homologs of *SFBB*.
 474 During the evolution of SI in Prunus, SLFLs may lose their function in S
 475 haplotype-specific interaction, and may recruit SFB for S haplotype-specific
 476 interaction. All together, these results suggested that PperSLFL1-3 have appropriate
 477 characteristics to be the GI.

478 Matsumoto (2012, 2016) showed that SFB and PavSLFLs interact with a
 479 Skp1-like1 homolog that is proposed to be a component of the SCF complex involved
 480 in the polyubiquitination of proteins targeted for degradation. By Y2H experiment, we
 481 have known that PperSLFL1-3 could interact with PperSSK1 and participate in the
 482 formation of SCF complex. It is possible that the PperSLFL1-3 would participate in
 483 the degradation of S-RNase proteins in a process as with that SLF/SFBB proteins are
 484 involved in the degradation of non-self S-RNase proteins (Kubo et al., 2010; Kakui et
 485 al., 2011; Williams et al., 2014; Kubo et al., 2015). In vitro ubiquitination analysis, we
 486 found that PperSLFL1-3 could make all the PperS-RNase proteins in this study tag
 487 the polyubiquitin chain (Fig. 6). According to the result, we suspect the SLFL proteins
 488 act as GIs to target all S-RNases with no allelic specificity in pollen.

489 In conclusion, our results suggested that PperSLFL1-3 were a subunit of SCF

complexes, recognized all S-RNases taken up into pollen tube and mediated polyubiquitination of S-RNases. Because loss-of-function of SFB results in pollen-part SC of peach unlike that in Japanese pear, the role of SLFL genes in the SI has been the focus of attention. The Y2H assay and activity of β -galactosidase assay showed that there was a strong interaction between PperSLFL1-3 and PperSSK1 and PperS-RNases. We thought that when S-RNases were taken up into the pollen tube, SFB would recognize self S-RNase and protect it by some kind of motification, and SLFL proteins could not recognize and target it. Cytotoxic effect of self S-RNase arrests pollen tube growing. When the SFB mutated, the ‘protection’ on self S-RNase disappeared, SLFL proteins target S-RNase and tag polyubiquitin chain on it, S-RNase could be degraded, and the pollen tube continue to grow to complete fertilization. This model is needed to be carefully tested, and further studies are needed to clarify the mechanism of self-incompatibility in *Prunus*.

503

504

505 **Figure legends**

506 **Fig. 1 Characterization and expression patterns of Chinese peach S genes**

507 (A) PCR analysis and schematic diagrams of the S2/2m-RNase in ‘Guang He Tao’.
508 The red line represents the mutation site of the S2-RNase. (B) Schematic diagram of
509 Chinese peach SFBs. The black arrows indicate the transcriptional orientations of the
510 genes. The red vertical bars indicate the stop codon, and the red numbers represent the
511 length of the encoding frame. The black boxes in SFB1m and SFB2m represent the

512 inserted fragment, and the gray boxes represent the same fragment of the gene as the
 513 inserted fragment. The black boxes represent the same fragments at both ends of the
 514 inserted fragment. (C) Schematic diagrams of the location of S2-RNase, SFB2m and
 515 SLFLs at the S-locus. The directions of the arrows represent the transcriptional
 516 orientations of S2-RNase, SFB2m and SLFLs. The middle parts in the red box
 517 represent the introns. (D) Tissue-specific expression analysis of *S1-RNase*, *S2-RNase*,
 518 *S2m-RNase* and *S4-RNase*, *SFB1m*, *SFB2m*, *SFB4m* and *SLFLs*. Total RNA from
 519 different organs was extracted and used as template for cDNA synthesis.

520 **Fig. 2 Aligment of the deduced amino acid sequences of PperSLFL1, PperSLFL2**
 521 **and PperSLFL3.** The three PperSLFLs sequences of Peach aligned using DNAMAN.
 522 The F-box domain is marked by purple line above, and the FBA domain is marked by
 523 black line above.

524 **Fig. 3 Yeast two-hybrid (Y2H) analysis to investigate interaction between**
 525 **S-RNases, PperSFBs, PperSLFLs, PperSSK, PperCUL1 and PperRbx1.** (A)
 526 and (E) Y2H assays and the activity of β -galactosidase assay of the interaction
 527 between PperSLFLs and S-RNases, PA1 (ppa011133m, a T2-RNase in *Prunus*
 528 *persica*). (B) Y2H assays and activity of β -galactosidase assay of interaction between
 529 PperSSK1, PperRbx1 and PperCUL1. (C) and (D) Y2H assays and the activity of β -
 530 galactosidase assay of interaction between PperSSK1 and PperSFBs and PperSLFLs.
 531 Empty vector was used as negative control; SV40 and p53 was used as positive.
 532 AD-activation domain; BD-DNA binding domain.

Fig. 4 Bimolecular fluorescence complementation (BiFC) analysis of interactions
between PperSLFLs with S-RNases Construct pairs of PperSLFLs-YFPN,
S-RNases-YFPC, YFPN and YFPC were transiently co-expressed in *Nicotiana*
tabacum leaves. Fluorescence is indicated by the YFP signal. Merged images of YFP
as well as bright field images are shown. PperSLFLs-YFPN and S-RNases-YFPC
were co-injected with empty vector respectively as negative control. Scale bars = 10
μm.

Fig. 5 Immunoblot detection of S-RNases and F-box proteins *E.coli* expressing
S-RNases were detected by a polyclonal antibody. The polyclonal antibody against
the recombinant S2-RNase was raised in rabbit and the antibody detected not only
S2-RNase but also other allelic S-RNases without allelic specificity. *E.coli* expressing
F-box proteins were detected by commercial mouse monoclonal antibody. 10 μg of
total proteins were loaded in each lane.

Fig. 6 In vitro detection of ubiquitinated S-RNases Each of the lanes was loaded
with 10 μg protein. The ubiquitination of different PperS-RNases was analyzed with
the presence of PperSSK1, PperCUL1, PperSLFL1-3 and Ub. 10 μg of PperSLFL1-3
proteins was added to each lane, respectively. The lanes without His-tagged S-RNase
were used as negative control.

Supplemental data

Supplemental Fig. S1 The distribution of 37 peach varieties in China The arrows
in the figure represent the evolutionary direction of peach in China. The red shade

represents the origin of Chinese peach, and the gray shades represent the secondary center of origin of Chinese peach, and the purple circles represent various Chinese peach population.

Supplemental Fig. S2 Identification for the S genotypes of 36 peach varieties except Guang He Tao The PCR were performed with DNA extracted from leaves as template and primers Pru-C2/Pru-C4R for S₁ and S₂, and S₄ specific primers.

Supplemental Fig. S3. Phylogenetic trees of CDSs of S locus F-box and deduced amino acid sequences of Skp1-like proteins and cullin-like proteins. (A) A neighbor-joining tree was constructed from 64 S locus F-box genes from apple(*Malus domestica*; MdSFBBs), pear(*Pyrus × bretschneideri*; PbSFBBs), *Pyrus pyrifolia*; PpSFBBs), sweet cherry(*Prunus avium*; PavSFBs PavSLFLs), almond(*Prunus dulcis*; PdSFBs and PdSLFLs), plum(*Prunus mume*; PmSFBs and PmSLFLs, *Prunus salicina*; PsSFBs), apricot(*Prunus armeniaca*; ParSFBs), sour cherry(*Prunus cerasus*; PcSFB26), peach(*Prunus persica*; PperSFBs and PperSLFLs) and *Prunus speciosa* (PspSFB1). (B) Skp1-like proteins and cullin-like proteins were used to construct NJ trees. The deduced amino acid sequences of Skp1-like proteins were from *Arabidopsis thaliana*(AtSKPs), *Antirrhinum hispanicum*(AhSSK1), *Prunus tenella*(PtSSK1), *Petunia integrifolia*(PiSKP1,PiSKP3), *Pyrus × bretschneideri*(PbSKP1), *Malus domestica*(MdSSK1-2), *Prunus avium*(PavSSK1) and *Prunus persica*(PperSSK1). The deduced amino acid sequences of cullin-like proteins were from *Arabidopsis thaliana*(AtCUL1-3), *Prunus avium*(PavCUL1A, PavCUL1B), *Petunia integrifolia* (PiCUL1C), *Vitis vinifera*(VvCUL1-1,VvCUL1-2), *Nicotiana*

577 *tabacum*(NtCUL1-1) *Prunus tomentosa*(PtCUL1), *Prunus mume*(PmCUL), *Pyrus* ×
578 *bretschneideri*(PbCUL1, PbCUL1-1), *Malus domestica*(MdCUL1-1,MdCUL1-2) and
579 *Prunus persica* (PperCUL1). NJ trees were generated with 1000 bootstrap replicates.

580 **Supplemental Fig. S4 Yeast two-hybrid assay and for the interactions between**
581 **PperSFBs and S-RNases** (A) Yeast two-hybrid assay for the interactions between
582 PperSFBs and S-RNases. (B) The activity of β -galactosidase assay for the interactions
583 between PperSFBs and S-RNases. Each of the combinations was selected 10 yeast
584 plaques and then divided into 3 portions. Each portion was cultured and the activity of
585 β -galactosidase was measured separately. (C) Constructs of AD::box, AD::box-V1,
586 AD::V1-V2 and AD::HV_a-HV_b.

587 **Supplemental Fig. S5 Yeast two-hybrid assay and BiFC assay for the interaction**
588 **between S-RNases and portions of PperSFB2** (A) Yeast two-hybrid assay for the
589 interaction between S-RNases and portions of PperSFB2. Various combinations of
590 AD and BD fusions are tested for their growth on SD/-Leu/-Trp/-His/-Ade media. (B)
591 BiFC assay for the interaction between S-RNases and portions of PperSFB2.
592 Fluorescence is indicated by the YFP signal. Merged images of YFP as well as bright
593 field images are shown. Scale bars = 10 μ m.

594 **Supplemental Table 1 Thirty seven wild and local genotypes of Chinese peach**

595

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607

608 **Contributions**

609 T. L. and D. M. designed the study; Q. C., D. M. and Z. G. performed the experiment.
610 W. L., X. D. Q. Y. and Y. L. contributed reagents/materials. T. L. and D. M. wrote the
611 paper. All authors read and approved the final manuscript.

612

613 **Competing financial interests**

614 The authors declare no competing financial interests.

615

616

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A

B

C

D

F-box motif

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A

B

C

D

E

YFPC

A

Anti-S-RNase

B

Anti-MBP