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

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15 **Title**

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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*

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

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

Introduction

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

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

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

94 reveals that S^5 pollen is normally accepted by S^1, S^3 and S^4 pistils (Sassa et al., 2011).
95 The previous fruit set analyses shows that S^5 pollen is normally compatible with S^2
96 and S^9 pistils and incompatible with S^5 pistils (Kajiura et al., 1967; 1969; 1974). On
97 the contrary, in *Prunus*, a truncated SFB protein or lacked the SFB gene can confer
98 pollen-part self-compatibility (SC) (Ushijima et al., 2004; Sonneveld et al., 2005;
99 Hauck et al., 2006; Tsukamoto et al., 2006). These findings suggest that
100 S-RNase-based GSI seems to consist of two types in which the mode of action of
101 pollen S is different, a 'self recognition by a single factor' system and a 'non-self
102 recognition by multiple factors' system (Kakui et al., 2011), and the S-RNase-based
103 GSI of *Prunus* represents 'self recognition by a single factor', in which the cytotoxic
104 effect of non-self S-RNases in pollen tubes is inactivated by a 'general inhibitor'
105 while the self S-RNase is specifically protected by a 'blocker' molecule and degrades
106 RNA of self-pollen to arrest tube growth (Luu et al., 2001; Sonneveld et al., 2005).
107 Although the 'general inhibitor' is a hypothetical protein and had been considered to
108 be SLFLs in *Prunus avium* (Matsumoto and Tao, 2016), and in peach, whether the
109 'general inhibitor' is SLFLs as with in *Prunus avium* needs to be studied.

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

to the substrate protein to make ubiquitination of substrate proteins (Wu et al., 2000; Zheng et al., 2002; Deshaies and Joazeiro, 2009). SLF/SFBB is shown to be a compoment of the SCF complex to detoxified non-self S-RNases. In petunia, SLF was showed to form the SCF complex with Skp1-like and Cul1-p in pollen(Zhao et al., 2010; Entani et al., 2014; Liu et al., 2014), and in Maloideae, SFBB was also shown to form SCF complex which targeted selectively S-RNase and polyubiquitinated it in vitro (Yuan et al., 2014).

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

Materials and Methods

Plant Material

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

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

DNA and RNA Extraction

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

PCR for S Genotype Analysis

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

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

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

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

Tissue-specific expression analysis

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

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

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

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

Bimolecular fluorescence complementation (BiFC) analysis

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

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

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

Purification of Peptide Tagged Recombinant Proteins

His-tagged proteins were purified as previously described (Meng et al., 2014). The mature peptide of the S-RNases without signal peptide were cloned into the pEASY-E1 vector (From TransGen Biotech Company) and transformed into the *E. coli* strain BL21 plysS (DE3) (From TransGen Biotech Company). The cells were inoculated into LB medium containing 100μg/ml ampicillin and incubated for about 3h at 37 °C in a shaker at 200 rpm. Once the cell suspension OD_{600} reached about 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into medium with final concentration of 0.2 mM to induce protein expression. The cell suspension was incubated for 10-12 h at 16°C in the shaker at 180rpm. His-tagged fusion proteins were purified using Ni-NTA His Binding resin (Novagen, USA) as previously described (according to the manufacturer's instructions of Ni-NTA His Bind Resins, Novagen). The full-length coding sequences of pollen-expressed *PperSFB1m*, *PperSFB2m*, *PperSFB2*, *PperSFB4m PperSLFL1*, *PperSLFL2* and *PperSLFL3* were cloned into pMAL-c5x vector, which is designed to generate maltose-binding protein (MBP) fusion proteins. Similarly, the *PperCUL1*, *PperSSK1* and *PperRbx1* were cloned into pGEX4T-1 vector, which is designed to produce glutathione S-transferase (GST) fusion proteins. All the GST-fusion proteins and MBP-fusion proteins were purified using glutathione resin and maltose, as previously described (Yuan et al.,

2014).

In Vitro Ubiquitination Analysis of S-RNase

In vitro ubiquitination assays were performed as previously described (Yang et al., 2009; Yuan et al., 2014). The reaction mixture containing 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 5 mM HEPES, 2 mM adenosine triphosphate (ATP), 0.05% Triton X-100, 10 mM creatine phosphate, 1 unit of phosphokinase, 10 μg ubiquitin, 50 nM E1 (UBA6, *Petunia hybrida*), 1 mM PMSF, 850 nM E2 (UBH6, *P. hybrida*), and aliquots of the recombinant proteins GST-PpSSK1, GST-CUL1, GST-Rbx1 and any MBP-SLFL at 30 °C for 2 h. Mixtures were immunoblotted using an anti-S-RNase antibody (From Beijing ComWin Biotech Company).

Results

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

For this study, we collected 37 Chinese peach varieties which represent local cultivars in 18 provinces/municipalities in China (Supplemental Table 1). These are thought to represent the evolutionary paths from the origin in central China (Tibet, Yunnan and Guizhou provinces) to the northwest of China (Shanxi province), then to the southwest of China and finally to the coastal and Xinjiang provinces (Cao et al., 2014) (Supplemental Fig. S1). Only four *S*-haplotypes *S*1, *S*2, *S*2m and *S4*, were detected from 36 peach varieties except Guang He Tao (Supplemental Fig. S2) (Supplemental Table 1), and they had previously been reported (Tao et al. 2007). The

S genotype of 18 varieties, including Da Hong Pao, were *S2S2* genotype and 9 varieties, including Hunchun Tao, were *S1S2* genotype, while 3 varieties were of the S_2S_4 genotype (Feicheng Bai Li 10, Feicheng Bai Li 17 and Feicheng Hong Li 6) (Supplemental Table 1). The *S2-RNase* in 6 varieties was observed to contain a nucleotide substitution (G--A), which results in the conversion of the sixth conserved cysteine residue to a tyrosine in the *Prunus* C5 domain (Fig. 1A). This was named as *S2m-RNase*. *S2-RNase* and *S2m-RNase* were also identified from the original species Guang He Tao, which indicating that the mutation of *S2-RNase* had occurred before the formation of Chinese peach cultivars. Interestingly, we found that two *S-RNases* cloned from Guang He Tao lacked two introns (Fig. 1A). The 4 *S-RNases* were only expressed in pistil (Fig. 1D).

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

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

According to the sequences of *S-locus F-box-likes (SLFLs)* in peach genome (Genome Database for Rosaceae, http://www.rosaceae.org/), the primers were designed to clone the six *SLFL* genes. Finally, only three *SLFL* genes were cloned from pollen cDNA and their expression was pollen-specific (Fig. 1D). We named the three F-box genes as *PperSLFL1*, *PperSLFL2* and *PperSLFL3*, respectively. *PperSLFL1* located at about 47kb downstream of *PperS2-RNase* and the translation direction was opposite to that of *S2-RNase*; *PperSLFL2* located at about 26kb downstream of *S2-RNase* and *PperSLFL3* located at about 1.3kb upstream of *S2-RNase*, and the translation directions of the both F-box genes were the same as that of *S2-RNase*. The three *PperSLFL* genes did not have introns (Fig. 1C). The *PperSLFL1* and *PperSLFL2* showed pollen-specific expression, similar to the *PperSFB* genes. Despite the fact that *PperSLFL3* transcripts were detected in both leaves and styles, its expression was the most in pollen (Fig. 1D). The identity of the predicted amino acid sequences of PperSLFL1, PperSLFL2 and PperSLFL3 was 52.45%, while the alignment of the predicted amino acid sequences of PperSLFL1 with PperSLFL2 and PperSLFL3, respectively, the identity was 31.33% and 31.72%, respectively. The 3 *PperSLFL* genes showed low sequence identity with each other and with *PperSFB*. All the *PperSLFL* proteins contained the basic F-box domain and 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 *SFBBs* and *Prunus SLFL* genes clustered together and diverged into two secondary lineages. *Prunus SLFL* genes were more closely related to Pyrus and Malus *SFBB* 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' (*S1S2*) 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' (*S1S2*) 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

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

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

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

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

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

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

In order to test whether *S*-RNases could be ubiquitinated by SCF^{SLFL} in vitro, commercial His-UBA6 was used as the ubiquitin-activating (E1) and His-UBH6 was used as ubiquitin-conjugating enzyme (E2). Purified MBP-PperSLFL1-3, 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 Pper*SFB1m* 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 Pper*SLFL1-3*, 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,

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

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

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

490 complexes, recognized all S-RNases taken up into pollen tube and mediated
491 polyubiquitination of S-RNases. Because loss-of-function of SFB results in
492 pollen-part SC of peach unlike that in Japanese pear, the role of SLFL genes in the SI
493 has been the focus of attention. The Y2H assay and activity of β-galactosidase assay
494 showed that there was a strong interaction between PperSLFL1-3 and PperSSK1 and
495 PperS-RNases. We thought that when S-RNases were taken up into the pollen tube,
496 SFB would recognize self S-RNase and protect it by some kind of motification, and
497 SLFL proteins could not recognize and target it. Cytotoxic effect of self S-RNase
498 arrests pollen tube growing. When the SFB mutated, the 'protection' on self S-RNase
499 disappeared, SLFL proteins target S-RNase and tag polyubiquitin chain on it,
500 S-RNase could be degraded, and the pollen tube continue to grow to complete
501 fertilization. This model is needed to be carfully tested, and further studies are needed
502 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

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

558 **Supplemental Fig. S2 Identification for the S genotypes of 36 peach varieties**
559 **except Guang He Tao** The PCR were performed with DNA extracted from leaves as
560 template and primers Pru-C2/Pru-C4R for S_1 and S_2, and S_4 specific primers.

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

577 *tabacum*(NtCUL1-1) *Prunus tomentosa*(PtCUL1), *Prunus mume*(PmCUL), *Pyrus×*
578 *bretchneideri*(PbCUL1, PbCUL1-1), *Malus domestic*(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::HVa-HVb.

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 μm.

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.

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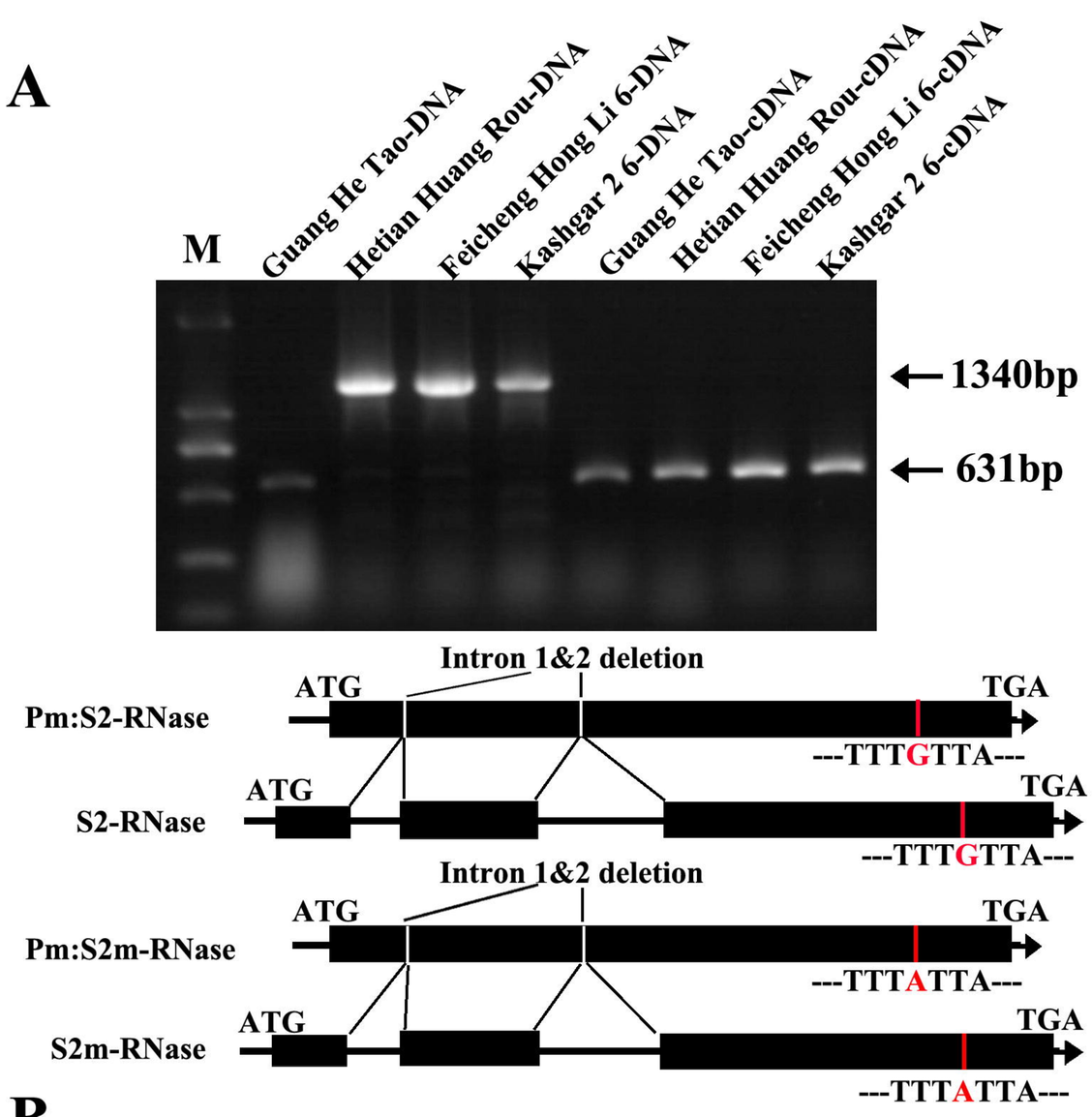
A



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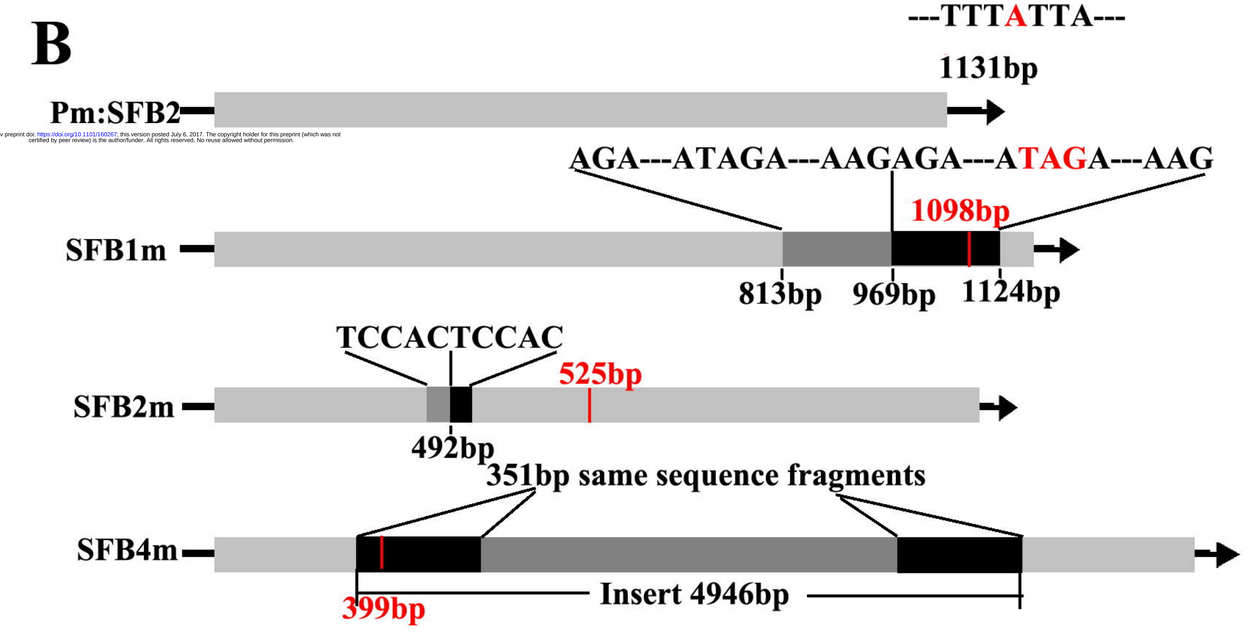

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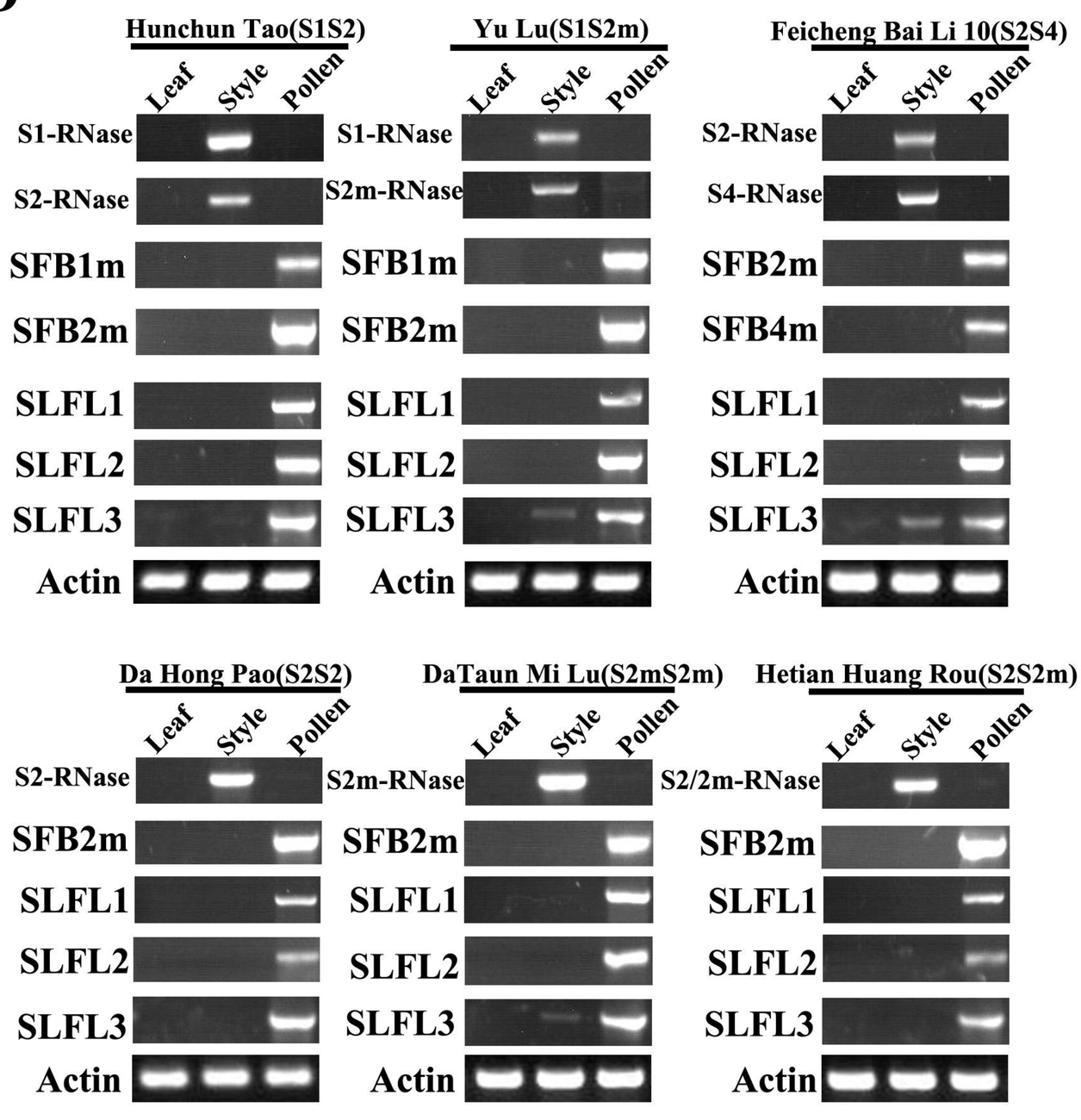

F-box motif

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PperSLFL1   MATLSKFSEDMMGN. ILSRLPPKSLMRFRCVLKSWHDLIDKPSFVDQHLSTSMDNKVTSS   59
PperSLFL2   ......MSEEMVLH. ILSRLPSKSLMRFKCVRKSWYTMINDPMFVERHLSNSMHNKRSTS   53
PperSLFL3   ......MWEEMALHRILPRLPSKSLIRFKCVRKSWYNLIDSPTFVENHLSISMHKIHST.   53
Consensus         e m   il rlp ksl rf cv ksw  i  p fv  hls sm

PperSLFL1   TCVLLKHNVLTDPTIKDDEKAVRATLFNPDSNQRDILLSSLNLGSLVDDGLEIENHVVPP  119
PperSLFL2   .CILVNRFLHSDTSTGEKELGFSFLYFCNDYDDD.....EHKVNSFVEDIKFPFSSGGQR  107
PperSLFL3   .CVLFNRFIYSDANTGEKELAFSFLYLRNDYDDDA....ENNVNFVVEDIKFPLSSR.QY  107
Consensus    c l       d       e           d                 v d

PperSLFL1   PMRGYALSLEISGSCDGLICLNTFNSEDIVLCNPALEEYRVLPKSCILLPPRVPRQVEEN  179
PperSLFL2   SGFEVIESLSMIGQCDGIVCLCDRRD.NIVLCNPAIKELKLLPKSCLP............  154
PperSLFL3   IGLGVIESLCMAGHCHGIVCLSESNS.NLVLCNPGIKKIKLLPKSCLP............  154
Consensus          sl  g c g  cl          vlcnp       lpksc

PperSLFL1   EDDDYYEEDDDDEIESNPKCVGFGYDPNSKDYKVVRAAQFVSGVFTQ....HPSKVEVYS  235
PperSLFL2   ..............QLIQCAVGFGYDPKSKDYKIHRISCDGEEIYDQRLVFFPPRVEIYT  200
PperSLFL3   ..............DWGCAVGFGYDPKSKDYKVSRIASYQADIYGDGLIP.PPRVEIYT  199
Consensus           vgfgydp skdyk   r                 p  ve y
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FBA

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PperSLFL1   LAADTWREIP...VDIQPHGSLNPSYQMYFNGFFYWIAYWT..............EERNV  278
PperSLFL2   LTTDSWREIKNNYLETENTFFWPEYFEMYWKGICYWLGYEQPKEFESYFDRLEDEKKKTM  260
PperSLFL3   VSTDSWREIKNNSLETEATFFCPDYFQMYFQGICYWVGFEQPKQSVEY....EDEEQKPM  255
Consensus       d wrei                  my  g  yw

PperSLFL1   LLSFDMSEEVFHDIALPESGP.....DAYEYTS.........IAVWKDSLVLLLTSPVENE  324
PperSLFL2   IFLFDTGDEVSHNILLPDCLY...EAAVYRFDM.......RILLWNESVALFGLDSFGT   309
PperSLFL3   VFLFYTGDEIFHNILLPDSFYTYEDGSSYAYEMSYIMYTDLRIILWNGVIALFGFNRFSA  315
Consensus       f     e  h i l p           y             i  w     l

PperSLFL1   APKTLDLWVLDEELKGAKGLWTKHLAIGPLAKGVEAPLVFWKDEELLMVTTNGDVVNYSL  384
PperSLFL2   FVECYGLWVLDD.FDGAKGSWTRHCTFESVID.IKRVLAFWNNDEILLVDGCGNIVSYNL  367
PperSLFL3   FPDSYGVWVLD...DGAKGSWTQHLTFEPLVG.IKRVLEFWKSDEILMVTEDGDIVSYNL  371
Consensus           wvl d    gakg wt h              l fw  e l v  g  v y l

PperSLFL1   DTQMLKHVPRHGLGEPTNIQAVPYVNSIVSIKPGNKIESI..                    424
PperSLFL2   GTNNHKNLLIYTENL.CRVETIVYVNTLVPIMGGNKLESVDK                    408
PperSLFL3   ATEKLKNLPRNSS...SDFETIVYVNSLVSITGRQQA....                     405
Consensus    t   k              yvn  v i
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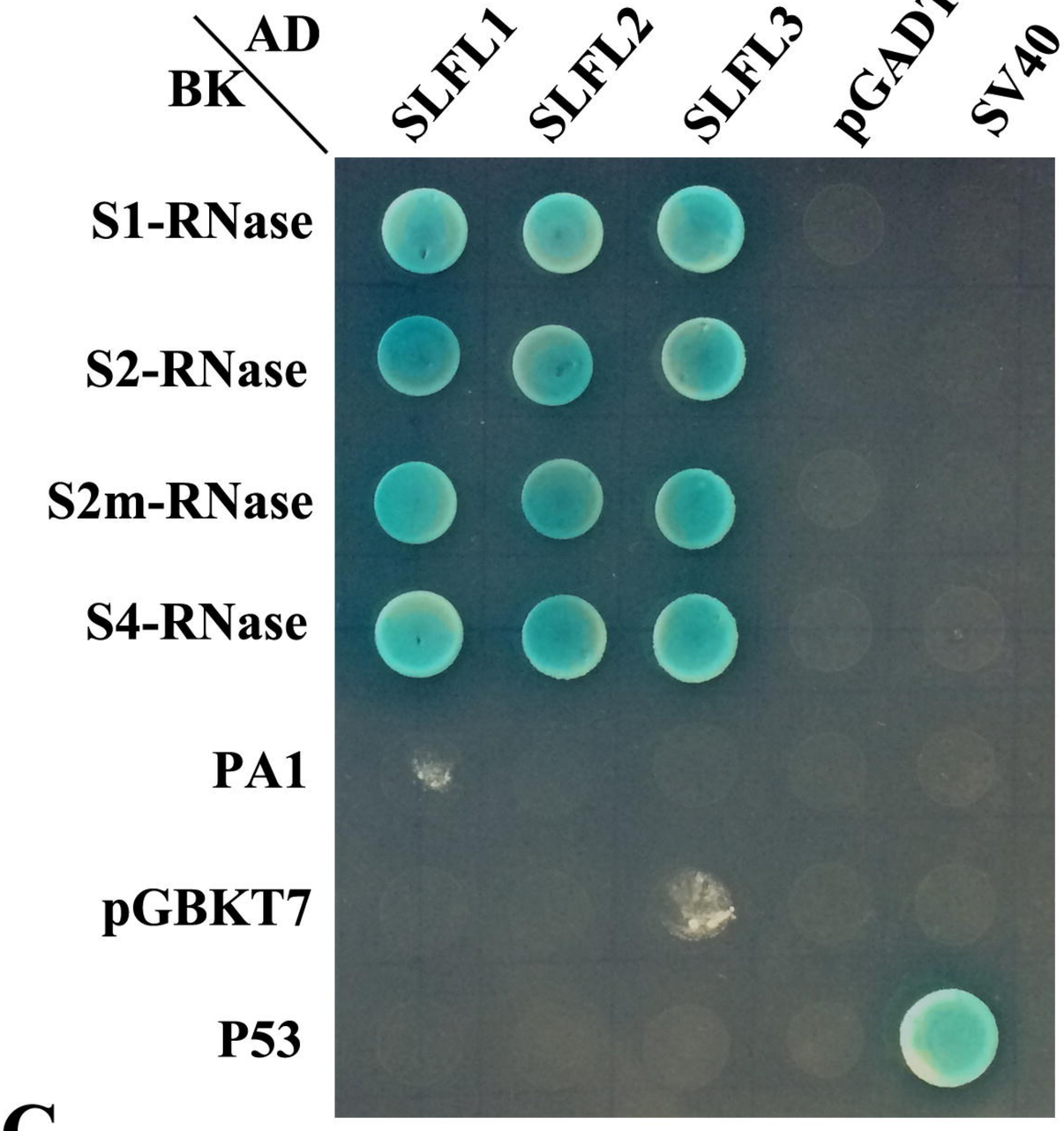
A



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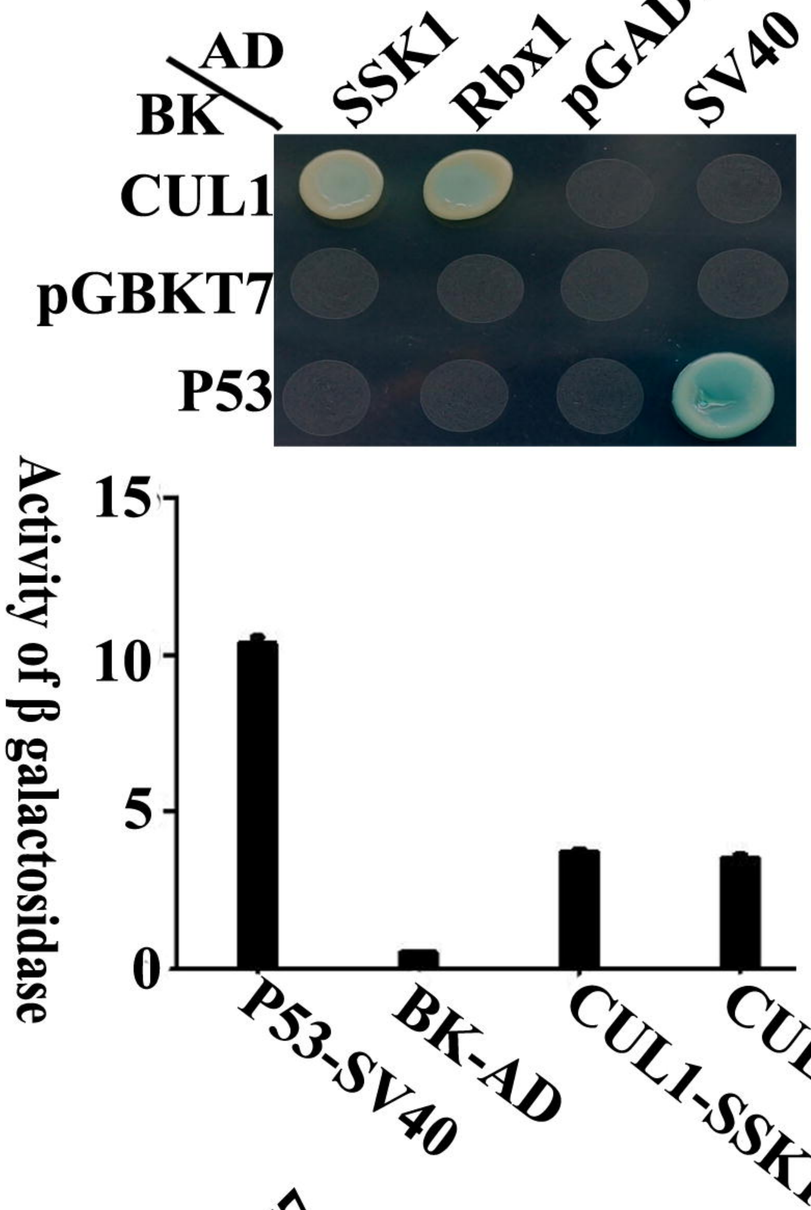

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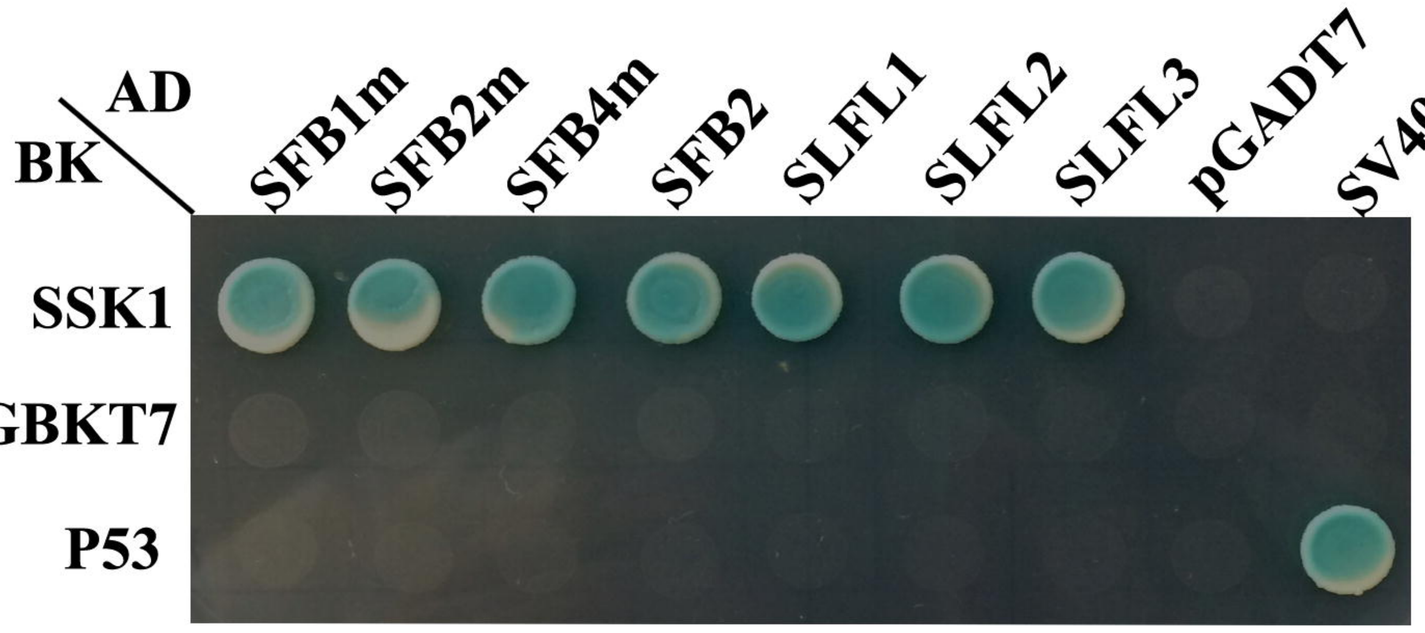

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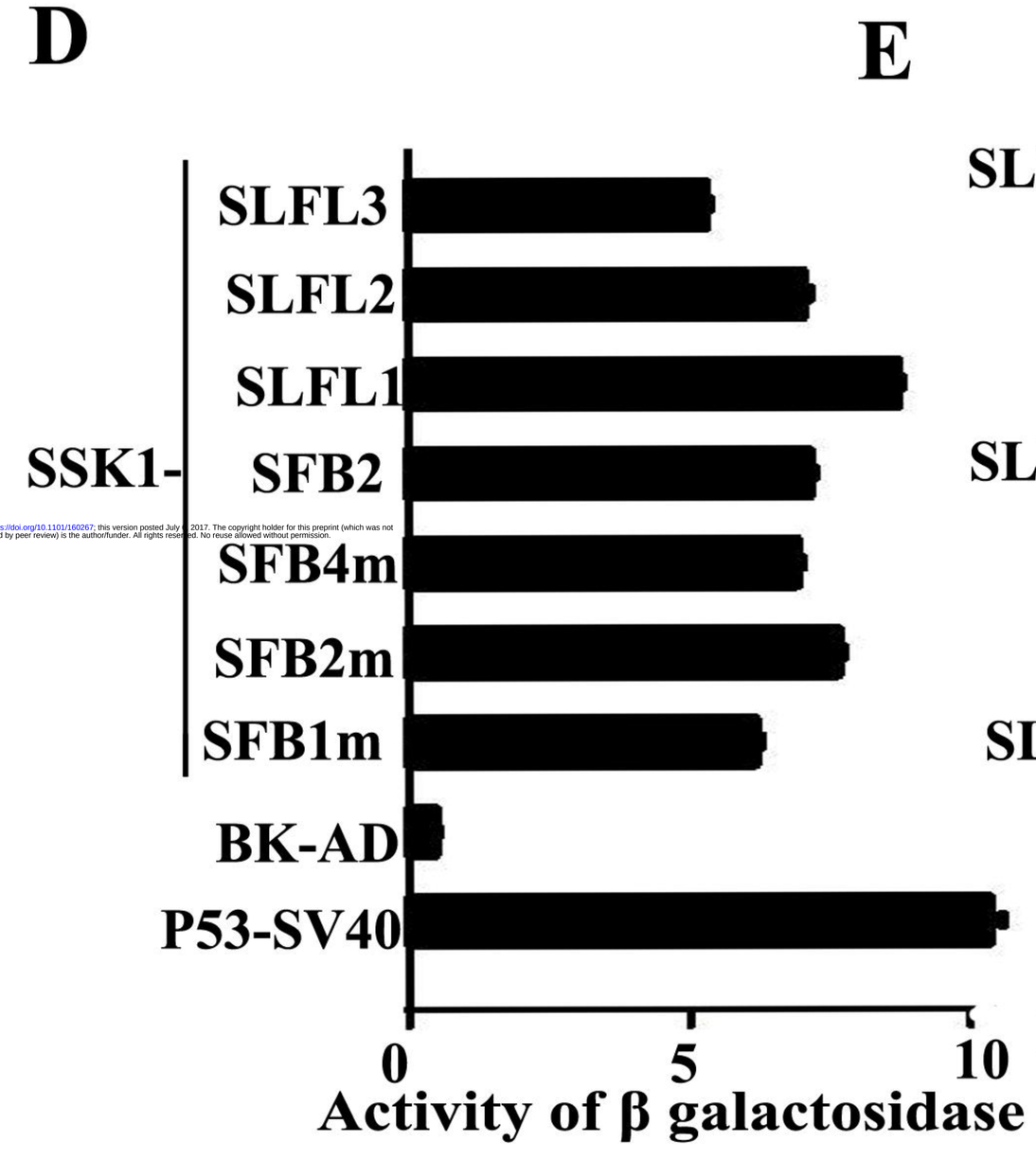

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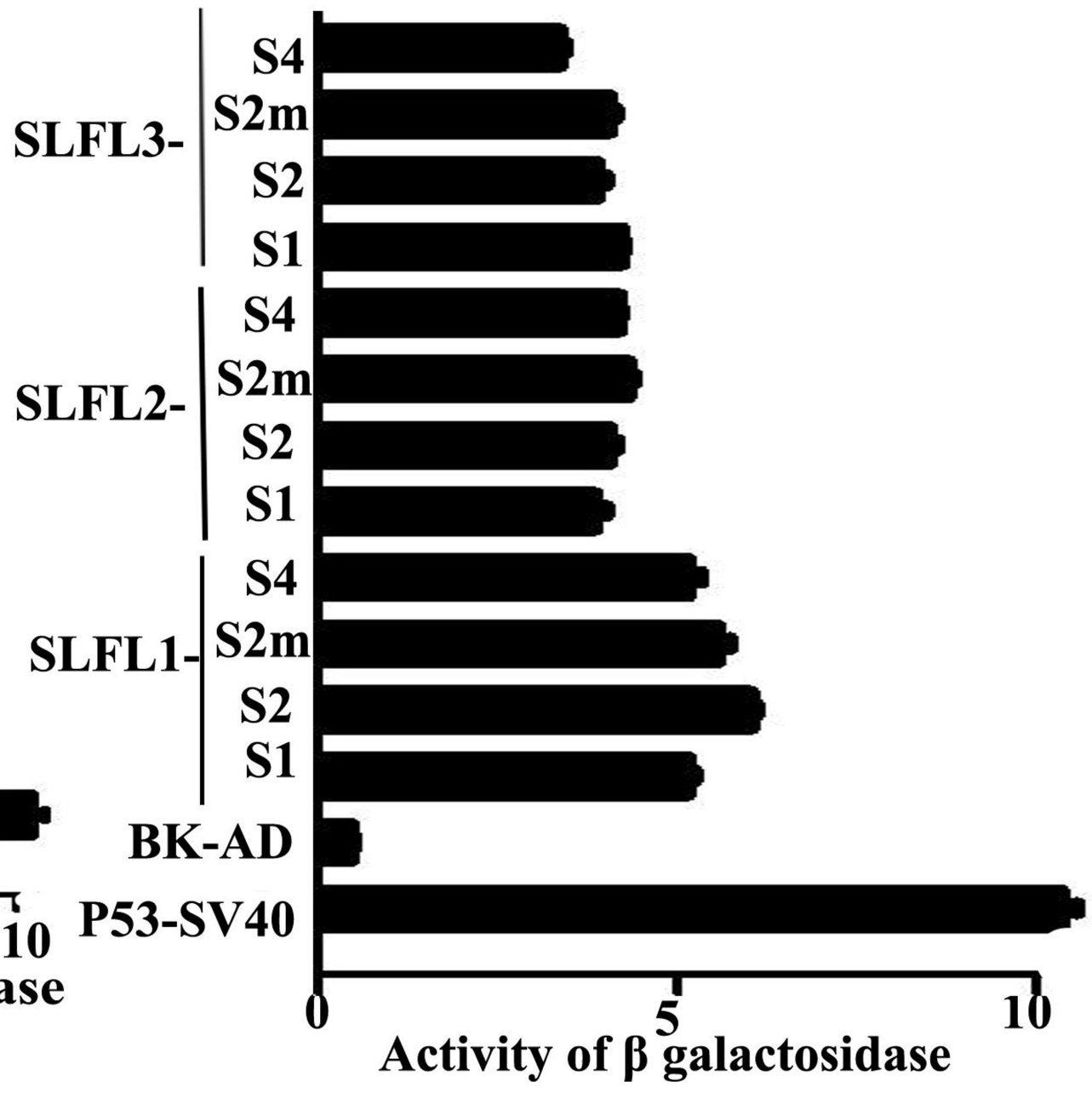


YFPC

S1-RNase | S2-RNase | S2m-RNase | S4-RNase | YFPC

YFP | Merge

YFPN

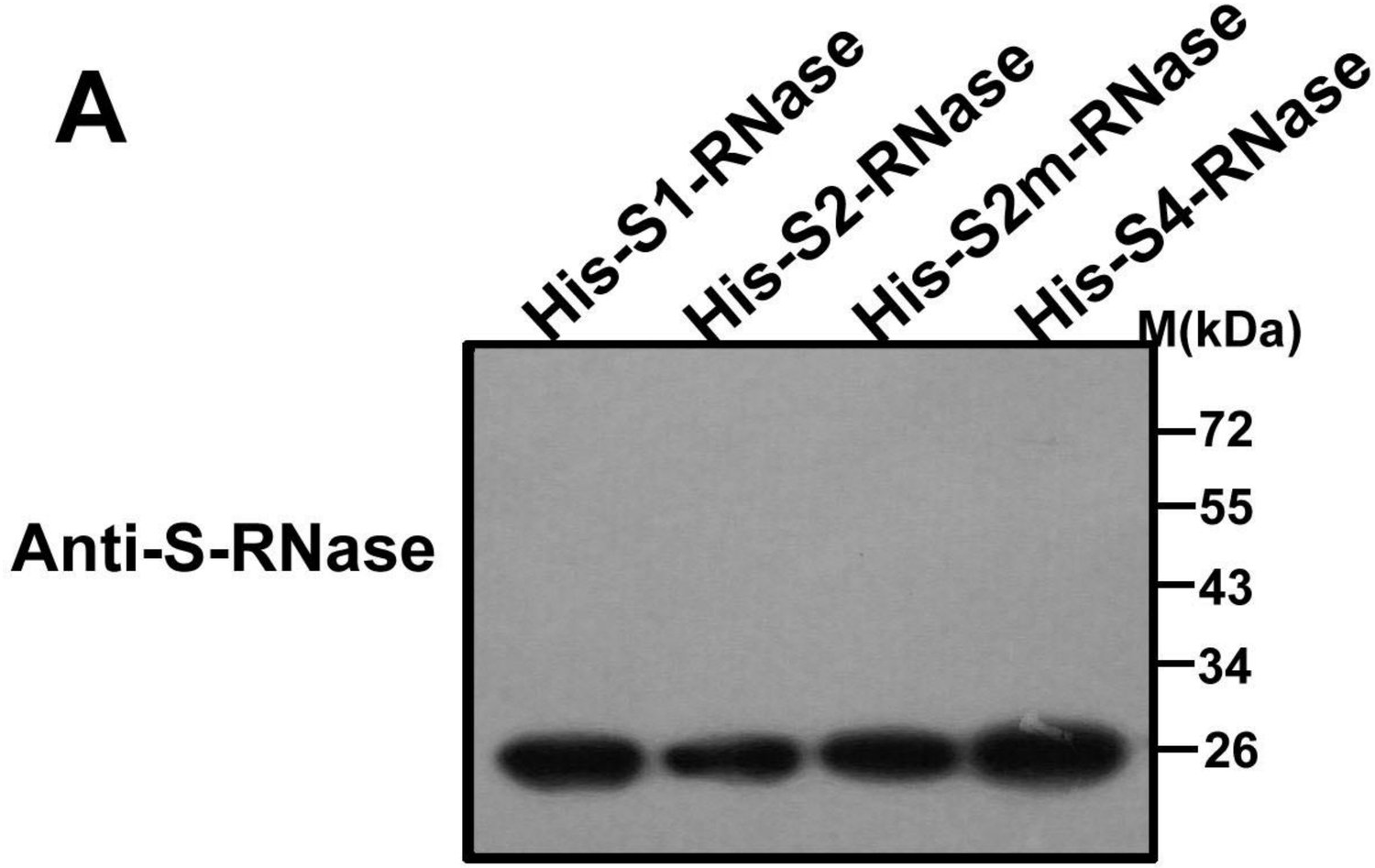
SLFL1

SLFL2

SLFL3

YFPN

A



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