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Identification of a canonical SCF^{SLF} complex involved in S-RNase-based self-incompatibility of *Pyrus* (Rosaceae)

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Abstract S-RNase-based self-incompatibility (SI) is an intraspecific reproductive barrier to prevent self-fertilization found in many species of the Solanaceae, Plantaginaceae and Rosaceae. In this system, *S-RNase* and *SLF/SFB* (*S-locus F-box*) genes have been shown to control the pistil and pollen SI specificity, respectively. Recent studies have shown that the SLF functions as a substrate receptor of a SCF (Skp1/Cullin1/F-box)-type E3 ubiquitin ligase complex to target S-RNases in Solanaceae and Plantaginaceae, but its role in Rosaceae remains largely undefined. Here we report the identification of two pollen-specific SLF-interacting Skp1-like (SSK) proteins, PbSSK1 and PbSSK2, in *Pyrus bretschneideri* from the tribe Pyreae of Rosaceae. Both yeast two-hybrid and pull-down assays demonstrated that they could connect PbSLFs to PbCUL1 to form a putative canonical SCF^{SLF} (SSK/CUL1/SLF) complex in *Pyrus*. Furthermore, pull-down assays showed that the SSK proteins could bind SLF and CUL1 in a cross-species manner between *Pyrus* and *Petunia*. Additionally, phylogenetic analysis revealed that the SSK-like proteins from Solanaceae, Plantaginaceae and Rosaceae form a monoclade group, hinting their shared evolutionary origin. Taken together, with the recent identification of a canonical SCF^{SFB} complex in *Prunus* of the tribe Amygdaleae of Rosaceae, our results show that a conserved canonical SCF$^{SLF/SFB}$ complex is present in Solanaceae, Plantaginaceae and Rosaceae, implying that S-RNase-based self-incompatibility shares a similar molecular and biochemical mechanism.

Keywords SSK · SLF · SCF complex · S-RNase · Self-incompatibility · *Pyrus*

Chi Xu and Maofu Li contributed equally to this work.

Accession numbers: HE802069 (*PbSLF3-S$_{34}$*), HE802070 (*PbSLF6-S$_{21}$*), HE802072 (*PbSSK1*), HE802071 (*PbSSK2*), and HE802075 (*PbCUL1*).

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Abbreviations

SI	Self-incompatibility
SLF	*S*-locus F-box
SFB	*S*-haplotype-specific F-box
SFBB	*S*-locus F-box brother
SSK	SLF-interacting Skp1-like
SCF	Skp1-Cullin1-F-box
SBP1	S-RNase-binding protein1

Introduction

Self-incompatibility (SI) is an intraspecific prezygotic reproductive barrier found in many flowering plants, which prevents inbreeding and promotes outcrossing by rejecting self (genetically related) pollen but accepting non-self (genetically unrelated) pollen for fertilization (De Nettancourt 2001; Franklin-Tong 2008). In most cases, SI is controlled by a single polymorphic locus, named the *S*-locus. It contains at least two tightly linked genes, the pistil *S* and the pollen *S*, and the interaction of their protein products determines the self-/non-self-specific recognition between pistil and pollen (Takayama and Isogai 2005; Zhang et al. 2009; Chen et al. 2010; McClure et al. 2011). Among the Solanaceae, Plantaginaceae and Rosaceae, the pistil and pollen *S* encode a pistil-expressed ribonuclease (S-RNase) and a cluster of pollen-expressed *S*-locus F-box (SLF/SFB) proteins, respectively (Anderson et al. 1986; Sassa et al. 1992; Ushijima et al. 1998; Lai et al. 2002; Entani et al. 2003; Ushijima et al. 2003; Yamane et al. 2003; Zhou et al. 2003; Qiao et al. 2004a; Sijacic et al. 2004; Sassa et al. 2007; Kubo et al. 2010).

Recent studies have shown that the SLF functions as a component of a multi-subunit SCF (Skp1/Cullin/F-box)-type E3 ubiquitin ligase, but two alternative SLF-containing complexes have been proposed. In *Antirrhinum hispanicum* (Plantaginaceae) and *Petunia hybrida* (Solanaceae), a canonical SCF^{SLF} complex has been detected, based on the identification of a pollen-specific SLF-interacting Skp1-like1 (SSK1) protein, which could serve as an adaptor to bridge SLF to CUL1, as observed in the canonical SCF complexes that are composed of Skp1, Cullin1, an F-box protein and Rbx1 (Zheng et al. 2002; Huang et al. 2006; Zhao et al. 2010; Chen et al. 2012). Interestingly, SSK1 appears to represent a novel type of Skp1-like proteins, with a distinguished feature having a unique disordered coil tail at the C-terminus following the conventional residues "WAFE" found in most plant Skp1-like proteins (Gagne et al. 2002; Risseeuw et al. 2003). In addition, the SSK1 family appears to form a monoclade among Skp1-like proteins (Huang et al. 2006; Zhao et al. 2010). However, in *Petunia inflata*, an alternative non-canonical SCF^{SLF} complex is suggested and appears to consist of three subunits: SBP1 (S-RNase-binding protein1) (Sims and Ordanic 2001), CUL1 and SLF. In this E3 complex, the constitutively expressed SBP1 is proposed to play a dual role: serving as an adaptor to bridge SLF to CUL1 similar to Skp1 and also acting like Rbx1 to connect the novel E3 complex to an E2-like protein (Hua and Kao 2006). Although further studies are required to define the precise roles of these two alternative SCF complexes in SI, two additional lines of evidence obtained from both *Antirrhinum* and *Petunia* support that SLFs function as substrate receptors in an SCF E3 ligase manner involving the ubiquitin–proteasome system. First, SLF could interact with S-RNases in a non-*S*-haplotype-specific manner and appears to exhibit a stronger interaction affinity to non-self S-RNase than self one and second, the ubiquitination of S-RNases has been observed, which could be blocked by proteasomal inhibitors (Qiao et al. 2004b; Hua and Kao 2006, 2008; Hua et al. 2007; Kubo et al. 2010).

However, it is unclear whether similar SLF-containing complexes are present in Rosaceae. Although the Solanaceae, Plantaginaceae and Rosaceae all employ S-RNase as the pistil *S* determinant and SLF/SFB as the pollen *S* determinant, SLF/SFB identified in *Prunus* from the tribe Amygdaleae of Rosaceae appears to function differently, as the deletion or alteration of *SFB* in *Prunus* is thought to lead to self pollen acceptance rather than rejection as found in the Solanaceae and Plantaginaceae (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006a; Tsukamoto et al. 2006; Vilanova et al. 2006). Therefore, *Prunus* SFB is proposed to function as a protector to prevent self S-RNase from detoxification rather than a destroyer to target non-self S-RNase for degradation as observed in Solanaceae and Plantaginaceae. Additionally, competitive interaction, a well known phenomenon observed in the Solanaceae and Plantaginaceae whereby the coexistence of two different pollen *S* alleles in a pollen results in the pollen SI breakdown, is not always observed in tetraploid *Prunus* (Entani et al. 1999; Golz et al. 1999, 2001; Hauck et al. 2006b; Xue et al. 2009). However, multiple related SFBBs (*S*-locus F-box brothers), the pollen *S* candidates identified in the tribe Pyreae (e.g., apple and pear) of Rosaceae, are assumed to play a similar role in SI response as SLFs in Solanaceae and Plantaginaceae, because the loss-of-function mutants of *SFBB* genes display a collaborative non-self recognition pattern, similar to *Petunia* (Sassa et al. 2007; Kubo et al. 2010; Minamikawa et al. 2010; Kakui et al. 2011; Okada et al. 2011). Furthermore, the tetraploids of this tribe exhibit competitive interaction (Crane and Lewis 1942; Adachi et al. 2009).

In this study, we first identified two pollen-specific SLF-interacting Skp1-like (SSK) proteins in *Pyrus bretschneideri* from the tribe Pyreae of Rosaceae, named PbSSK1 and

PbSSK2, which could serve as adaptors to bridge PbSLF to PbCUL1, together constituting a putative canonical SCF^{SLF} (SSK/CUL1/SLF) complex. Second, we demonstrated that SSK proteins interact with SLF and CUL1 in a cross-species manner between *Pyrus* and *Petunia*. Furthermore, we found that SSKs appear to share a single evolutionary origin. Collectively, our results, together with the recent identification of a canonical SCF complex in *Prunus* (Matsumoto et al. 2012), show that a conserved canonical SCF complex occurs in Solanaceae, Plantaginaceae and Rosaceae, supporting the notion that S-RNase-based self-incompatibility shares a similar molecular and biochemical mechanism.

Materials and methods

Plant materials

Pyrus bretschneideri cultivar "YaLi" ($S_{21}S_{34}$) and its spontaneous self-compatible mutants ("YanZhuang" and "JinZhui") (Li et al. 2009), "JinFeng" ($S_{19}S_{34}$) and "ZiSu" ($S_{19}S_{34}$) were obtained from Weixian Orchard, Hebei Province, China. Leaves, pollen, and pistils from buds were collected in liquid nitrogen and stored at −80 °C for later DNA and RNA isolation.

DNA gel blot analyses

Genomic DNA isolation was performed as described previously (Xue et al. 1996). DNA (10 μg) was digested with *Hin*dIII and *Eco*RI, separated on 0.8 % agarose gel and transferred onto Hybond-N+ membrane (Amersham, Buckinghamshire, UK). Prehybridization, hybridization and washing of the blot were performed as recommended by the manufacturers. Probes were labeled with ^{32}P by random priming using the Prime-a-Gene labeling system (Promega, Madison, WI, USA).

CAPS and dCAPS analyses

Genomic DNA of an F_1 population of 38 progeny from a cross "YaLi" ($S_{21}S_{34}$) × "JinFeng" ($S_{19}S_{34}$) was used as the template for PCR amplification of *PbSLF* genes. For *PbSLF6-S_{21}*, the PCR products were digested with *Kpn*I to detect the specific cleaved amplified polymorphic sequence (CAPS) band. For *PbSLF3-S_{34}*, derived CAPS (dCAPS; Neff et al. 1998) analysis was performed, and the PCR products were digested with *Spe*I to detect the specific band (Table S4). The products were separated on 1 or 4 % agarose gels and visualized by staining with ethidium bromide. The primers were listed in Table S2.

RT-PCR and RT-PCR/CAPS analysis

Total RNA was prepared as previously described (Lai et al. 2002) and digested with DNase I (TaKaRa, Dalian, China). The cDNA was produced using the SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and a poly-dT primer. The RT-PCR was performed to detect the expression patterns of *PbS_{21}-RNase*, *PbS_{34}-RNase*, *PbSSK1*, *PbSSK2* and *PbCUL1*. The RT-PCR/CAPS was conducted to detect the expression patterns of *PbSLF6-S_{21}* and *PbSLF3-S_{34}*. Gene-specific primers were listed in Table S2.

Yeast two-hybrid assays

The full-length coding sequences of *PbS_{21}-RNase* and *PbS_{34}-RNase* without signal peptides and the full-length coding sequences of *PbSSK1* and *PbSSK2* were cloned into *pGADT7* (Clontech, CA, USA), respectively, to produce fusion proteins with the GAL4 activation domain. The N-terminal (1-150 bp), C-terminal and full-length coding sequence of *PbSLF3-S_{34}* and *PbSLF6-S_{21}* and the full-length coding sequence of *PbCUL1* were separately introduced into *pGBKT7* (Clontech, CA, USA), to form recombinants with the GAL4 DNA binding domain. The various combinations of BD and AD vectors were co-transformed into yeast strain AH109 and grown on SD/-Leu-Trp medium at 30 °C for 3–4 days. The clones were subsequently grown on SD/-Ade-His-Leu-Trp medium at 30 °C for 7 days to test interaction.

GST pull-down assays

The full-length coding sequences of *PbSSK1*, *PbSSK2* and *PhSSK1* were separately cloned into *pGEX-6p-1*(GE Healthcare) and *pET-28b* (Novagen) to produce GST- and His_6-tagged fusion proteins. The full length of *PbSLF3-S_{34}*, *PbSLF6-S_{21}*, *PhSLF-S_1* and *PhSLF-S_3* with engineered N-terminal SUMO tag were separately cloned into *pET-30a* (Novagen) to generate His_6-SUMO-tagged fusion proteins. The DNA fragments encoding the PbCUL1-NTD (residues 1–383, with V345D and I349E) and PhCUL1-NTD (residues 1–379, with I341E and I345E) were cloned into *pGEX-6p-1* (GE Healthcare), respectively, to produce soluble GST-tagged fusion proteins, according to the approach described by Zheng et al. (2002).

All the fusion proteins were expressed in *E. coli* BL21 (DE3) at 16 °C. The His_6- and His_6-SUMO-tagged fusion proteins were purified using Ni–NTA (Novagen) and eluted with buffer containing 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 250 mM imidazole. The GST-tagged fusion proteins were purified using Glutathione Sepharose 4B (GE Healthcare) and eluted with buffer containing 25 mM Tris–HCl

(pH 8.0), 150 mM NaCl, 3 mM DTT and 15 mM reduced glutathione. Then the eluted proteins were dialyzed with buffer containing 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 3 mM DTT (wash buffer).

For SLF/SSK GST pull-down assays, an equal amount of GST-SSK and His_6-SUMO-SLF were mixed and incubated on ice for 3 h. Subsequently, the mixture was loaded onto Glutathione Sepharose 4B resin columns. After washing with wash buffer for five times, the proteins were eluted with wash buffer supplemented with 15 mM reduced glutathione. The elutes were separated by 12 % SDS-PAGE, transferred to PVDF membranes (Millipore) and probed with anti-His (Sigma-Aldrich) and anti-GST (Sigma-Aldrich) antibodies, respectively. Various combinations between SSKs and SLFs were detected. GST and His_6-SUMO were used as negative controls. For CUL1-NTD/SSK GST pull-down assays, the similar procedure was performed with GST-CUL1-NTDs as bait and His_6-SSKs as prey.

Alignment and phylogenetic analyses

Amino acid sequence alignments of the Skp1-like proteins were performed using ClustalW (Chenna et al. 2003). Secondary structure prediction was carried out using the SWISS-MODEL (http://swissmodel.expasy.org/). Phylogenetic analyses of the *SKP1-like*, *Cullin-like* and *SLF* genes based on deduced amino acid sequences were carried out using a neighbor-joining (NJ) method with 1,000 bootstrap replicates using MEGA version 5.0 (Tamura et al. 2011).

Results

Molecular identification of *SLF* alleles from *P. bretschneideri*

Previously, *S_{21}-RNase* and *S_{34}-RNase* were isolated from a self-incompatible "YaLi" cultivar (*$S_{21}S_{34}$*) of *P. bretschneideri* by Wu et al. (2007). To further characterize them, we performed RT-PCR and Southern blot analyses, and found that both are the pistil- and *S*-haplotype-specific genes (Fig. 1a, b).

To isolate the pollen *S* genes from *P. bretschneideri*, we carried out homology-based cloning based on the conserved sequences of the *SFBB* alleles identified in the tribe Pyreae, using the genomic DNA from "YaLi" (*$S_{21}S_{34}$*) as templates (Sassa et al. 2007). Two SLF candidates were obtained showing high amino acid sequence similarity to SFBB alleles from the tribe Pryeae. To detect whether these two *SLF* candidates are linked to *S-RNases*, CAPS and derived cleaved amplified polymorphic sequence (dCAPS) analyses were conducted using an F_1 population of 38 progeny from a cross "YaLi"(*$S_{21}S_{34}$*) × "JinFeng" (*$S_{19}S_{34}$*), and showed that one *SLF* candidate is linked to *S_{21}-RNase* and the other to *S_{34}-RNase* (Fig. S1). Furthermore, phylogenetic analysis of these two SLF candidates with SFBBs from *Pyrus pyrifolia* and *Malus domestica* showed that the *S_{21}*-haplotype-specific SLF candidate belongs to the type-6 clade and the *S_{34}*-haplotype-specific one to the type-3 clade (Fig. S2) (Kakui et al. 2011). Thus, they were named *PbSLF6-S_{21}* (*Pyrus bretschneideri S-locus F-box type-6*) and *PbSLF3-S_{34}* (*Pyrus bretschneideri S-locus F-box type-3*), respectively. In addition, RT-PCR/CAPS revealed that both *PbSLF6-S_{21}* and *PbSLF3-S_{34}* are specifically expressed in pollen as expected (Fig. 1b).

To examine whether PbSLFs (PbSLF3-S_{34} and PbSLF6-S_{21}) interact with PbS-RNases, we performed a yeast two-hybrid assay. The full-length *PbS-RNases* (*PbS_{21}-RNase* and *PbS_{34}-RNase*) were introduced into *pGADT7* vector as prey, and three versions of *PbSLFs* (*PbSLF3-S_{34}* and PbSLF6-*S_{21}*) were introduced into *pGBKT7* vector as bait (Fig. 1c). Each *PbS-RNase* prey vector was cotransformed with individual *PbSLF* bait vector into Ah109 yeast cells for a growth test on the selective medium SD/-Ade-His-Leu-Trp. As shown in Fig. 1d, PbS_{21}-RNase interacts with the C-terminal regions of both PbSLF3-S_{34} and PbSLF6-S_{21}, while PbS_{34}-RNase only interacts with the C-terminal region of PbSLF3-S_{34}, showing that PbS-RNases specifically interact with the C-terminal regions of PbSLFs without the F-box domain, rather than the full-length PbSLFs and their N-terminal regions containing the F-box domain, indicated that PbSLFs could serve as pollen *S* determinants to interact with PbS-RNases, and these interactions are dependent on the C-terminal regions of PbSLFs similar to that found in Solanaceae and Plantaginaceae (Qiao et al. 2004b; Hua and Kao 2006; Hua et al. 2007; Kubo et al. 2010).

Molecular isolation of PbSSKs

Previously, we identified a new type of Skp1-like protein, SSK1 (SLF-interacting Skp1-like1), in both *Antirrhinum* (Plantaginaceae) and *Petunia* (Solanaceae), which could serve as a specific adaptor that bound the pollen *S* SLF to form a putative canonical SCF^{SLF} complex functioning in SI responses (Huang et al. 2006; Zhao et al. 2010). To test whether such SSK1-like proteins also exist in *P. bretschneideri*, we carried out an annotation search in the Genome Database for Rosaceae (http://www.rosaceae.org/) and homology-based cloning, based on one distinct feature of SSK1: a unique C-terminus which composed of a 7–9 amino acid tail of disordered coil following the conventional terminal residues "WAFE" found in many plant

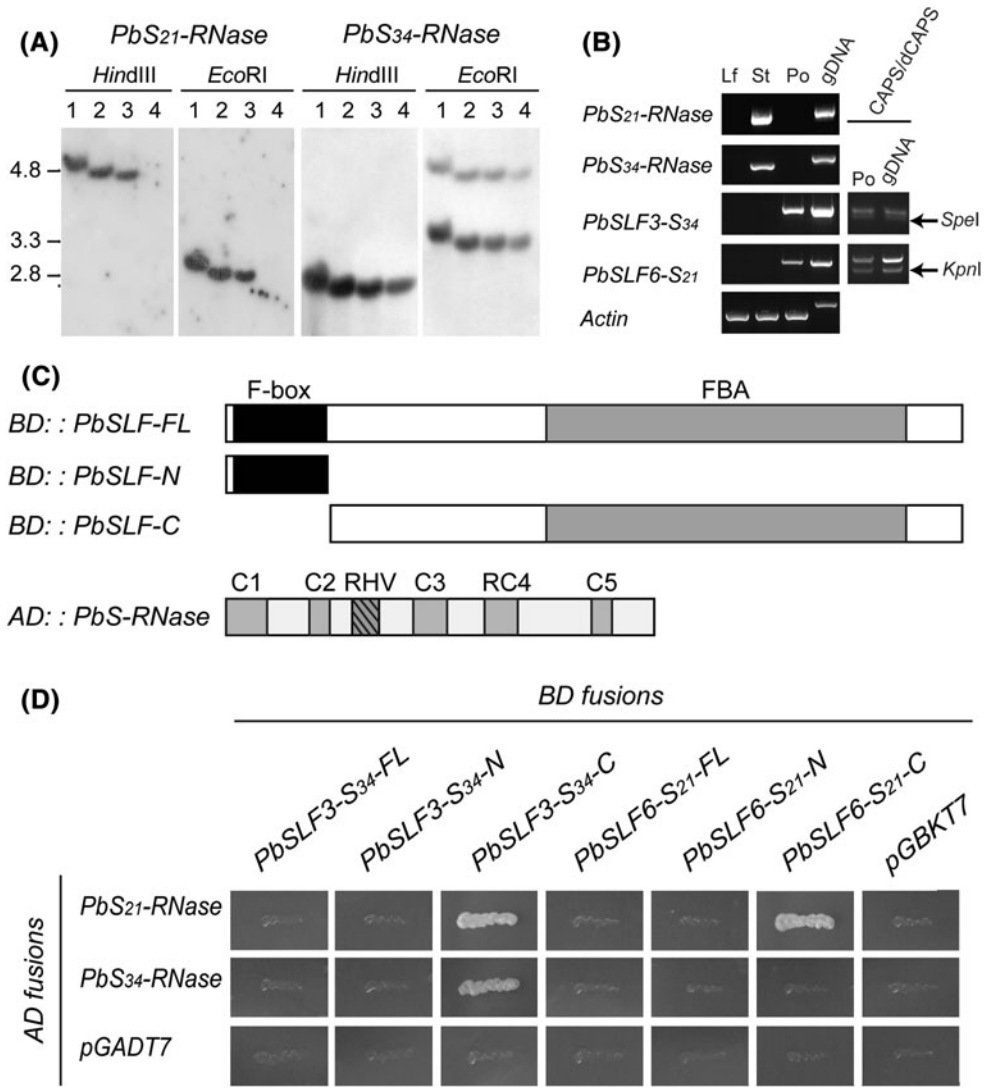

Fig. 1 Identification of SLFs interacting with S-RNases in *P. bretschneideri.* **a** Genomic DNA blot analyses of *PbS-RNases*. *Lanes 1–4* represent genomic DNA from four cultivars of *P. bretschneideri*, "YaLi" ($S_{21}S_{34}$), "YanZhuang" ($S_{21}S_{34}$), "JinZhui" ($S_{21}S_{34}$) and "ZiSu" ($S_{19}S_{34}$), respectively, which was separately digested by *Hind*III or *Eco*RI and probed by PbS_{21}*-RNase* (*left*) and PbS_{34}*-RNase* (*right*), respectively. "YanZhuang" and "JinZhui" were derived from spontaneous self-compatible sports from the "YaLi". The *numbers* indicate hybridizing fragment sizes in kilobase pairs. **b** Expression patterns of *PbS-RNases* and *PbSLFs* examined by RT-PCR or RT-PCR/CAPS. Total RNAs was extracted from leaf (*Lf*), style (*St*) and pollen (*Po*) of "YaLi" as indicated. The synthesized cDNA and genomic DNA (*gDNA*) were used as templates in RT-PCR or RT-PCR/CAPS. The "YaLi" *actin* gene was used as a positive control. **c** Schematic diagrams of *SLF* and *S-RNase* constructs used in yeast two-hybrid assays. FBA (F-box associated) represents a conserved domain found in SLF proteins; C1, C2, C3, RC4 and C5 indicate five conserved domains and RHV the hypervariable region in Rosaceae S-RNases. Signal peptides of PbS-RNases were deleted. **d** Yeast two-hybrid assays of PbSLFs with PbS-RNases. Cells of yeast strain AH109 containing various combinations of bait (BD fusion) and prey (AD fusion) were tested for their growth on selective medium SD/-Ade-His-Leu-Trp. The empty vectors *pGBKT7* and *pGADT7* were negative controls. The plates were photographed after 7 days incubation at 30 °C

Skp1-like proteins (Gagne et al. 2002; Risseeuw et al. 2003). Two *SSK1-like* genes were obtained, named *PbSSK1* (*P. bretschneideri SLF-interacting Skp1-like1*) and *PbSSK2* (*P. bretschneideri SLF-interacting Skp1-like2*), respectively.

PbSSK1 encodes a predicted polypeptide of 190 amino acid residues, which shares 39.0 % amino acid identity with AhSSK1 and 30.0 % with PhSSK1, respectively, and *PbSSK2* a predicted polypeptide of 201 amino acid residues, which shares 34.8 % amino acid identity with AhSSK1 and 29.4 % with PhSSK1, respectively. Meanwhile, both of them share 69.1 % amino acid identity with each other. As observed for AhSSK1 and PhSSK1, the two polypeptides encoded by *PbSSK1* and *PbSSK2* were predicted to contain eight α-Helices (H1–H8) and two β-Sheets (S1–S2) as found in the well-characterized human Skp1,

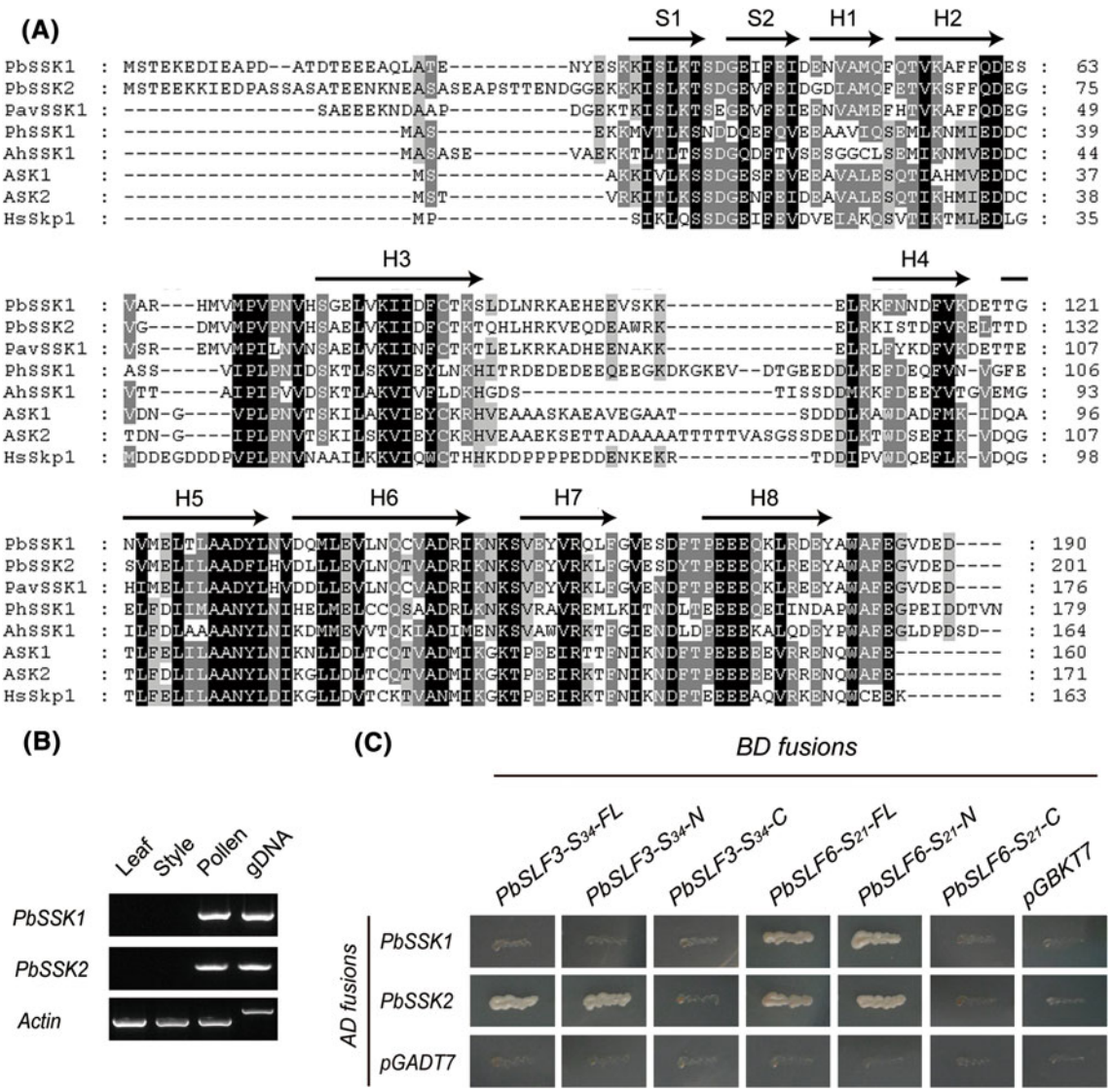

Fig. 2 Identification of the pollen-specific SSK proteins in *P. bretschneideri*. **a** Sequence alignment and structural elements of PbSSKs and several typical Skp1-like proteins. PavSSK1 (JQ322646), PhSSK1 (FJ490176), AhSSK1 (DQ355479), ASK1 (NM_115865) and ASK2 (NM_123584), and HsSkp1 (AAH20798) are from *P. avium*, *P. hybrida*, *A. hispanicum*, *A. thaliana*, and human, respectively. Residues of 80–100 % similarity are *shaded* in *black*, those of 60–80 % in *dark gray*, and 30–60 % in *light-gray*. *Numbers* show the positions of amino acid residues. The *arrows* represent the secondary structure. *S* β-sheet, *H* α-helix. **b** Expression patterns of *PbSSK1* and *PbSSK2* examined by RT-PCR. Total RNAs was extracted from three tissues of "YaLi" as indicated. The synthesized cDNA and genomic DNA (gDNA) were used as templates in RT-PCR. The "YaLi" *actin* gene was used as a positive control. **c** Yeast two-hybrid assays of PbSSKs with PbSLFs. The indicated combinations of bait (BD fusion) and prey (AD fusion) constructs were introduced into the yeast reporter strain AH109. Transformants were streaked on selective medium SD/-Ade-His-Leu-Trp and examined for growth. The empty vectors *pGBKT7* and *pGADT7* were negative controls. The plates were photographed after 7 days incubation at 30 °C

with a unique C-terminus composed of a 5 amino acid tail following the conserved terminal residues "WAFE" (Fig. 2a) (Schulman et al. 2000; Huang et al. 2006; Zhao et al. 2010). Also similar to *AhSSK1* and *PhSSK1*, both *PbSSK1* and *PbSSK2* were found to be specifically expressed in pollen (Fig. 2b). Then, we performed yeast two-hybrid assays to test whether PbSSK1 and PbSSK2 could interact with SLFs. The growth test in the yeast strain AH109 showed that the PbSSK1 interacts with the full-length PbSLF6-S_{21} or its N-terminal region with the F-box domain rather than that without the F-box, but no interaction was observed between PbSSK1 and PbSLF3-S_{34}; PbSSK2 interacts with the full-length and N-terminal regions of both PbSLF3-S_{34} and PbSLF6-S_{21}, except their

C-terminal regions lacking the F-box domain (Fig. 2c). Taken together, these results suggest that both PbSSK1 and PbSSK2 are the pollen-specific SLF-interacting Skp1-like proteins.

SSK proteins form a monophyletic clade among Skp1-like proteins

To examine the evolution of PbSSK proteins, we analyzed PbSSK1 and PbSSK2 by comparing with other plant Skp1-like proteins. Sequence alignment showed that PbSSK1 and PbSSK2 share higher amino acid identities to Skp1-like proteins from the rosaceous species. For example, PbSSK1 and PbSSK2 share 95.3 and 66.3 % amino acid identities with one Skp1-like protein from *M. domestica*, named MdSSK1-like1; 66.0 and 95.5 % with another Skp1-like protein from *M. domestica*, named MdSSK1-like2; 75.1 and 70.1 % with PavSSK1, a pollen-specific SFB-interacting Skp1-like protein recently identified in *Prunus avium* (Matsumoto et al. 2012); 66.9 and 65.7 % with a Skp1-like protein from *Fragaria vesca*, named FvSSK1-like1 (Table S1). To further confirm this finding, we analyzed the phylogenetic relationship of these Skp1-like proteins. PbSSK1 and PbSSK2 form a cluster with PavSSK1, FvSSK1-like1, MdSSK1-like1, MdSSK1-like2 and MdSSK1-like3, and this cluster is closely related to the cluster formed by AhSSK1 and PhSSK1, together to form a monophyletic clade (Fig. 3). Therefore, these results further reveal that SSK (SLF-interacting Skp1-like) proteins, as a new type of Skp1-like proteins, occur in not only Solanaceae and Plantaginaceae but also Rosaceae.

PbCUL1 interacts with PbSSKs in yeast

Next, we tried to isolate a Cullin1-like protein from *P. bretschneideri*, as the Skp1 protein usually acts as an adaptor to bridge the scaffold protein Cullin1 to an F-box protein in a typical SCF complex. Homology cloning identified a *Cullin1-like* gene from the pollen cDNA of "YaLi" cultivar of *P. bretschneideri*, named *PbCUL1* (*P. bretschneideri Cullin1*). *PbCUL1* encodes a predicted polypeptide of 753 amino acids showing 83.2 % amino acid identity with AtCUL1 and 80.8 % with PhCUL1, respectively, and was found to cluster together with *Arabidopsis thaliana* CULα group proteins in the phylogenetic tree (Fig. 4a) (Risseeuw et al. 2003; Marin 2009). Furthermore, RT-PCR revealed that *PbCUL1* appears to be highly expressed in pollen, lowly in styles, and barely in

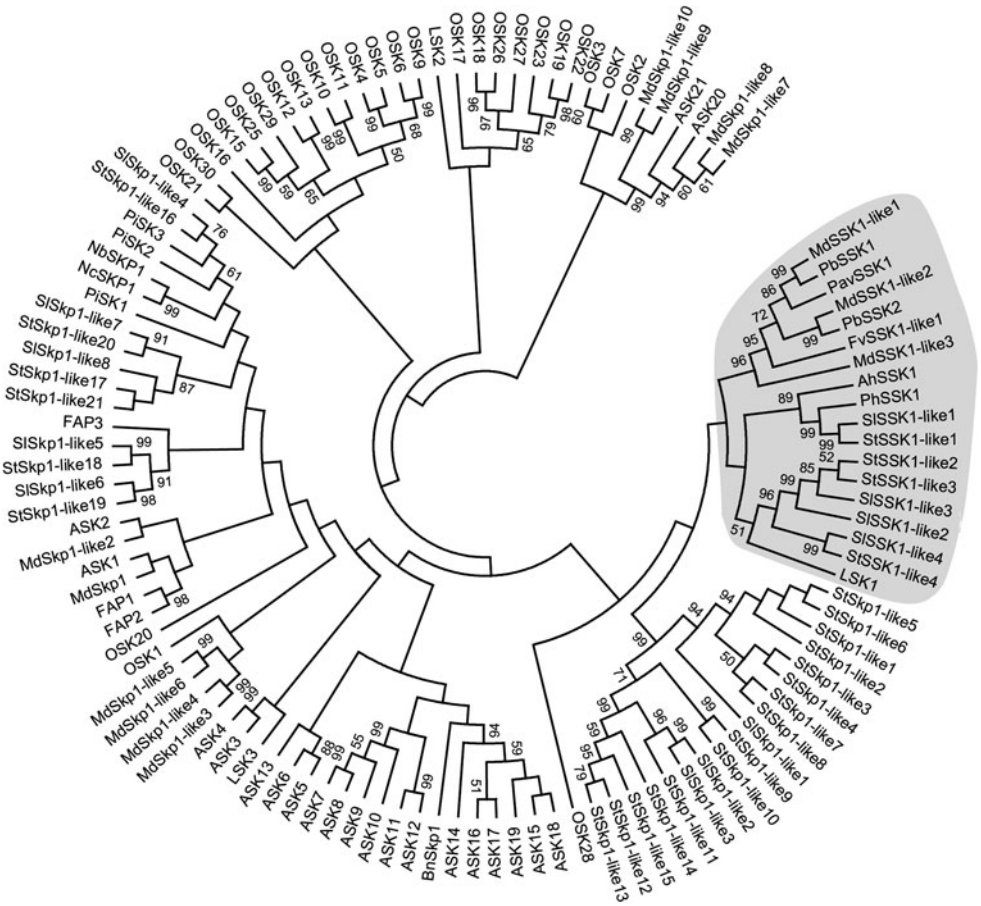

Fig. 3 An unrooted neighbor-joining phylogenetic tree of plant Skp1-like proteins. A total of 116 deduced Skp1-like proteins are from *Pyrus* (PbSSK1 and PbSSK2), *A. thaliana* (ASK1-ASK21), *Oryza sativa* (27 Skp1-like proteins previously surveyed by Kong et al. 2007), *Petunia* (PhSSK1 and PiSK1-PiSK3), *A. hispanicum* (AhSSK1 and FAP1-FAP3), *P. avium* (PavSSK1), *Brassica napus* (BnSkp1), *Lilium longiflorum* (LSK1–LSK3), *Nicotiana* (NbSkp1 and NcSkp1), *Solanum lycopersicum* (12 SlSkp1-like proteins), *Solanum tuberosum* (25 StSkp1-like proteins), *M. domestica* (13 MdSkp1-like proteins), and *F. vesca* (FvSSK1-like1). The unrooted neighbor-joining phylogenetic tree is generated with 1,000 bootstrap replicates, and bootstrap values of 50 % and above are given at branch nodes. The SSK1 family is highlighted in *gray shade*

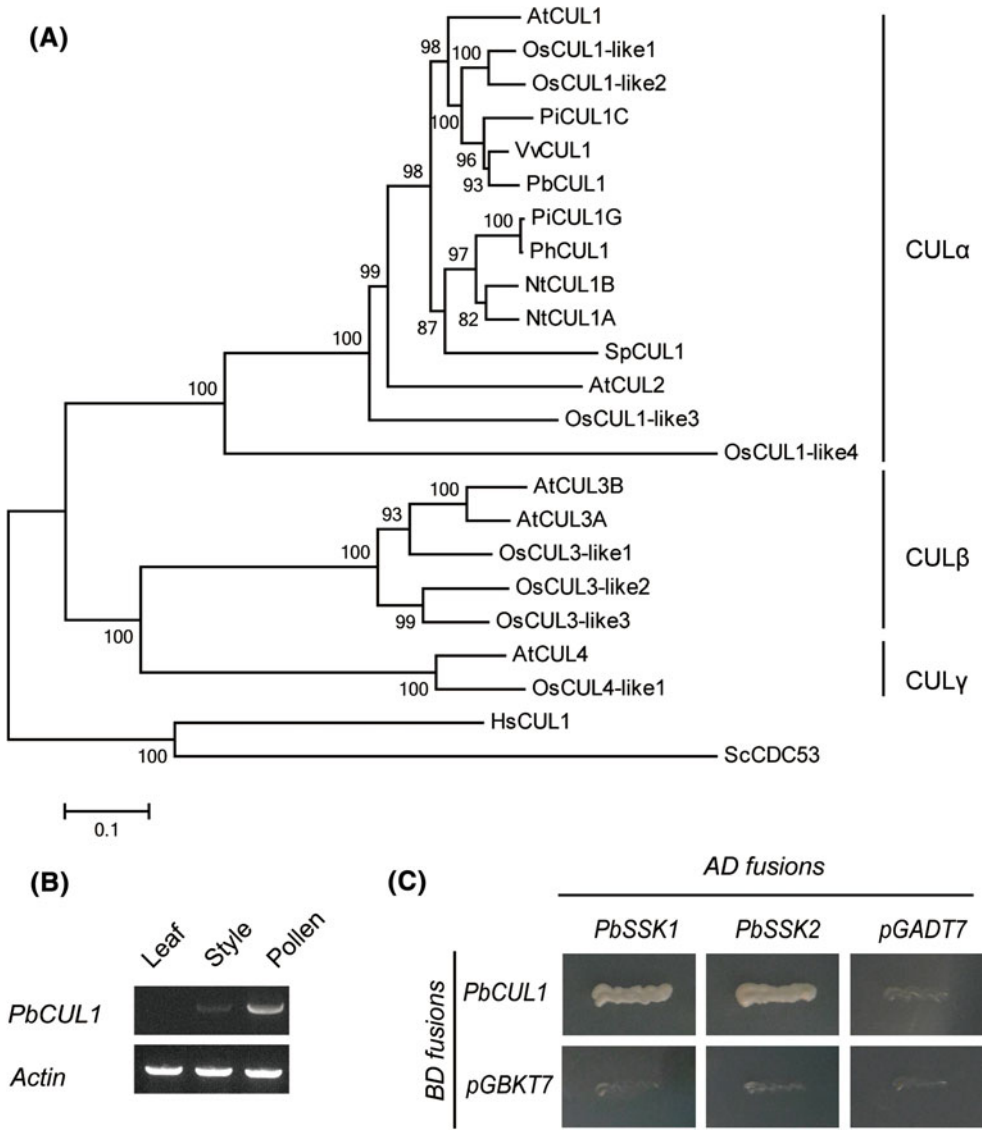

Fig. 4 Identification of PbCUL1 interacting with PbSSKs. **a** A neighbor-joining tree of PbCUL1 and other plant Cullin-like proteins. The deduced amino acid sequences are from *A. thaliana* (AtCUL1, NM_116491; AtCUL2, NM_100179; AtCUL3A, NM_102447; AtCUL3B, NM_105635; AtCUL4, NM_123990), *Oryza sativa* (OsCUL1-like1, LOCOs01g27150; OsCUL1-like2, LOC Os05g05700; OsCUL1-like3, LOC Os01g27160; OsCUL1-like4, LOC Os01g50980; OsCUL3-like1, LOCOs02g51180; OsCUL3-like2, LOCOs08g07 400; OsCUL3-like3, LOC Os04g55030; OsCUL4-like1, LOCO s03g57290), tobacco (NtCUL1A, AJ344533; NtCUL1B, AJ344533), tomato (SpCUL1, HQ610201), *Vitis* (VvCUL1, XM_002272159), and *Petunia* (PhCUL1, FJ490178; PiCUL1C, DQ250016; PiCUL1G, DQ250017). The neighbor-joining tree is generated with 1,000 bootstrap replicates, and bootstrap values of 50 % and above are given at branch nodes. Budding yeast Culα ScCDC53 (NM_001 180191) and Human CUL1 (NM_003592) are used as the outgroup. **b** Expression patterns of *PbCUL1* examined by RT-PCR. Total RNAs was extracted from three tissues of "YaLi" as indicated. The synthesized cDNA were used as templates in RT-PCR with gene-specific primer pairs. The "YaLi" *actin* gene was used as a positive control. **c** Yeast two-hybrid assays of PbSSKs with PbCUL1. The indicated combinations of bait (BD fusion) and prey (AD fusion) constructs were introduced into the yeast reporter strain AH109. Transformants were streaked on selective medium SD/-Ade-His-Leu-Trp and examined for growth. The empty vectors *pGBKT7* and *pGADT7* were negative controls. The plates were photographed after 7 days incubation at 30 °C

leaves (Fig. 4b). Then, we examined the potential interaction between PbCUL1 and PbSSKs by yeast two-hybrid assays. As shown in Fig. 4c, PbCUL1 could interact with both PbSSK1 and PbSSK2, suggesting the existence of the putative SCF^{SLF} (SSK/CUL1/SLF) complex in *P. bretschneideri*.

PbSSKs bridge PbSLF to PbCUL1 to form a canonical SCF complex

To further confirm the CUL1-SSK-SLF interaction, we first examined the interactions between PbSSKs and PbSLFs by GST pull-down assays. The GST-tagged full-length PbSSKs

(GST-PbSSK1/-PbSSK2) were used as bait against the His_6-SUMO-tagged full-length PbSLFs (His_6-SUMO-PbSLF3-S_{34}/-PbSLF6-S_{21}). As shown in Fig. 5a, both PbSSK1 and PbSSK2 could interact with PbSLF3-S_{34} and PbSLF6-S_{21}, though no interaction was observed between PbSSK1 and PbSLF3-S_{34} in yeast likely due to a technical limitation associated with the yeast two-hybrid system. Then, the GST-tagged N-terminal domain of PbCUL1 (GST-PbCUL1-NTD), responsible for binding Skp1 in a typical SCF complex (Zheng et al. 2002), was used as bait against the His_6-tagged full-length PbSSKs (His_6-PbSSK1/-PbSSK2). As shown in Fig. 5b, both PbSSK1 and PbSSK2 could interact with the N-terminal domain of PbCUL1. Together, these results further show that PbSSK1 and PbSSK2 could serve as specific adaptors to bridge PbSLF to PbCUL1 to form putative canonical SCF^{SLF} (SSK/CUL1/SLF) complexes in *Pyrus*.

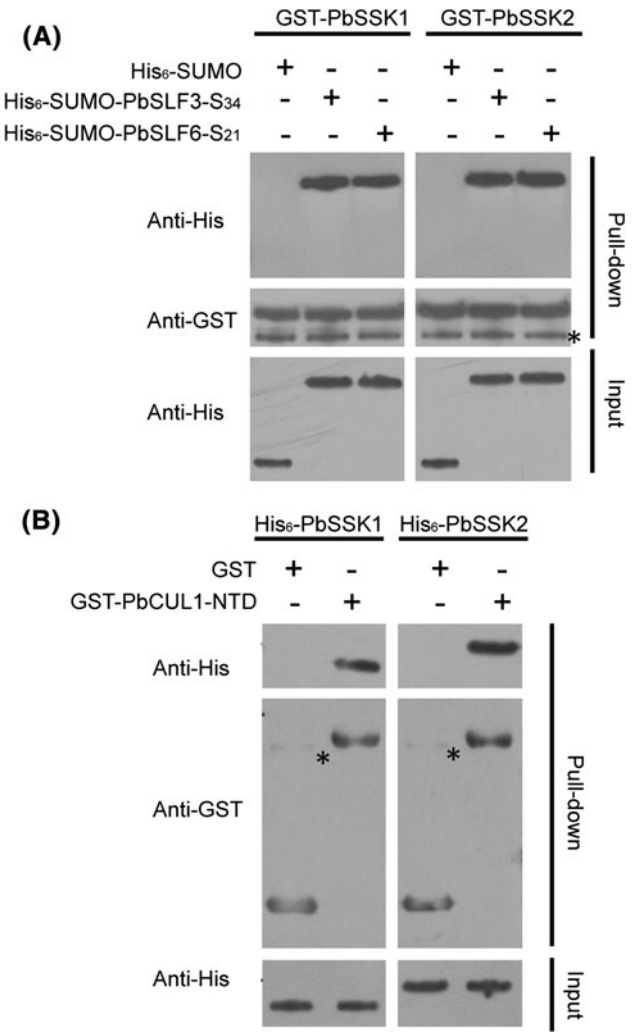

Fig. 5 The pollen-specific PbSSK proteins connect PbSLF to PbCUL1. **a** GST pull-down assays of PbSSKs with PbSLFs. Purified GST-tagged PbSSKs (GST-PbSSK1/PbSSK2) were used as bait against purified His_6-SUMO-tagged PbSLFs (His_6-SUMO-PbSLF3-S_{34}/PbSLF6-S_{21}). Bound proteins were examined with anti-His antibody. His_6-SUMO was a negative control. Five percent of purified His_6-SUMO and His_6-SUMO-tagged SLFs was loaded as input. The *asterisk* denotes a non-specific band detected by GST antibody. **b** GST pull-down assays of PbSSKs with PbCUL1. Purified GST-tagged N-terminal domain of PbCUL1 (GST-PbCUL1-NTD) was used as bait against purified His_6-tagged SSKs (His_6-PbSSK1/PbSSK2). Bound proteins were examined with anti-His antibody. GST was a negative control. Five percent of purified His_6-tagged PbSSKs was loaded as input. The *asterisk* denotes a non-specific band detected by GST antibody

Conserved interactions among the putative SCF^{SLF} complex subunits between *Pyrus* and *Petunia*

Considering the existence of the putative SCF^{SLF} (SSK/CUL1/SLF) complex in *Antirrhinum*, *Petunia* and *Pyrus*, we wondered whether each subunit in this SCF^{SLF} complex has a conserved role among them. To test this probability, we conducted cross-binding assays between *P. bretschneideri* and *P. hybrida*. We first tested the cross-interactions between SSKs and SLFs by GST pull-down assays. The GST-tagged full-length SSKs (GST-PbSSK1/-PbSSK2/-PhSSK1) were used as bait against the His_6-SUMO-tagged full-length SLFs (His_6-SUMO-PbSLF3-S_{34}/-PbSLF6-S_{21}/-PhSLF-S_1/-PhSLF-S_3). As shown in Fig. 6a, PhSSK1 interacts with both PbSLF3-S_{34} and PbSLF6-S_{21}, and PbSSK1 and PbSSK2 with both PhSLF-S_1 and PhSLF-S_3. Then, we tested the cross-interactions between SSKs and CUL1s by GST pull-down assays. The GST-tagged N-terminal domains of CUL1s (GST-PbCUL1-NTD/-PhCUL1-NTD) were used as bait against the His_6-tagged full-length SSKs (His_6-PbSSK1/-PbSSK2/-PhSSK1). As shown in Fig. 6b, PhSSK1 interacts with the N-terminal domain of PbCUL1, and PbSSK1 and PbSSK2 with the N-terminal domain of PhCUL1. These results suggest that a similar molecular and biochemical mechanism of S-RNase-based SI appears to be shared among Solanaceae, Plantaginaceae and Rosaceae.

Discussion

SLF physically interacts with S-RNase through forming a putative SCF^{SLF} complex in *P. bretschneideri*

In Solanaceae and Plantaginaceae, the pollen *S* determinant, *S*-locus F-box (SLF) protein, has been demonstrated to physically interact with both self and non-self S-RNases through forming a canonical SCF^{SLF} complex or a novel SCF-type E3 ubiquitin ligase complex (Qiao et al. 2004b; Hua and Kao 2006; Huang et al. 2006; Hua et al. 2007; Kubo et al. 2010; Zhao et al. 2010). In contrast, the biochemical role of SLF/SFB in Rosaceae is less well understood. In this study, we first isolated two pollen- and *S*-haplotype-specific SLF candidates from *P. bretschneideri* and then

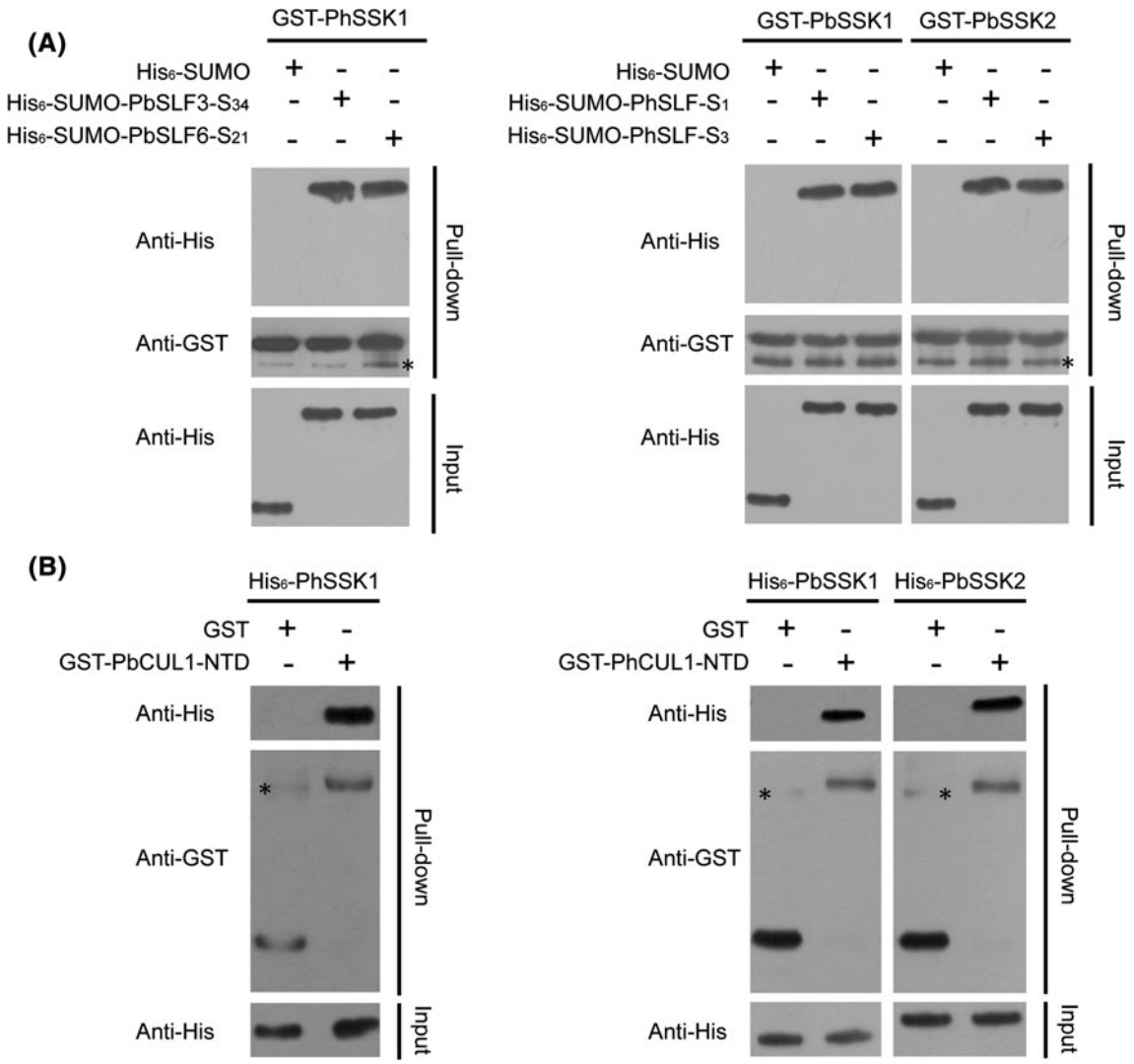

Fig. 6 Cross-species interaction of SSK, CUL1 and SLF between *P. bretschneideri* and *P. hybrida*. **a** Pull-down assays of SSKs with SLFs. Purified GST-tagged SSKs (GST-PbSSK1/PbSSK2/PhSSK1) were used as bait against purified His_6-SUMO-tagged SLFs (His_6-SUMO-PbSLF3-S_{34}/PbSLF6-S_{21}/PhSLF-S_1/PhSLF-S_3). Bound proteins were examined with anti-His antibody. His_6-SUMO was a negative control. Five percent of purified His_6-SUMO and His_6-SUMO-tagged SLFs was loaded as input. The *asterisk* denotes a non-specific band detected by GST antibody. **b** Pull-down assays of SSKs with CUL1s. Purified GST-tagged N-terminal domain of CUL1s (GST-PbCUL1-NTD/PhCUL1-NTD) were used as bait against purified His_6-tagged SSKs (His_6-PbSSK1/PbSSK2/PhSSK1). Bound proteins were examined with anti-His antibody. GST was a negative control. Five percent of purified His_6-tagged SSKs was loaded as input. The *asterisk* denotes a non-specific band detected by GST antibody

demonstrated that they could physically interact with PbS-RNases in yeast (Fig. 1 and S1). Second, this SLF-S-RNase interaction appears to be dependent on the C-terminal region of PbSLF, which is consistent with the role of the C-terminal domain of an F-box protein in a canonical SCF complex, responsible for binding specific substrate protein (Fig. 1). Third, we identified two pollen-specific SLF-interacting Skp1-like proteins, PbSSK1 and PbSSK2, structurally similar to AhSSK1 and PhSSK1 and cluster together to form a monoclade in the phylogenetic tree of plant Skp1-like proteins. Fourth, this SLF-SSK interaction seems to depend on the N-terminal F-box domain of PbSLF, as expected (Figs. 2, 3, 5a). Finally, we obtained a Cullin1-like protein with high expression level in pollen, PbCUL1, which could interact with both PbSSK1 and PbSSK2 (Figs. 4, 5b). These findings reveal that a putative SCF^{SLF} (SSK/CUL1/SLF) complex is also involved in the S-RNase-based SI system of the rosaceous tribe Pyreae, similar to that observed in Solanaceae and Plantaginaceae. In fact, a recent study in *Prunus* has shown that a similar SSK1-like protein mediates a putative SCF^{SFB} formation (Matsumoto et al. 2012). Together, these results support the view that a shared molecular and biochemical mechanism occurs in the three families possessing S-RNase-based SI.

SSK proteins, a class of conserved pollen factors involved in S-RNase-based SI

Besides S-RNase and SLF/SFB proteins, two conserved factors playing central roles in S-RNase-based SI, the SLF-interacting Skp1-like (SSK) protein appears to be another conserved SI factor because of its conserved presence in *Antirrhinum*, *Petunia*, *Pyrus* and *Prunus*. In fact, SSK1-like proteins are also found in apple (MdSSK1-like1/2/3), tomato (SlSSK1-like1/2/3/4, Solanaceae) and potato (StSSK1-like1/2/3/4, Solanaceae). In contrast, no apparent SSK1-like protein could be found in *Arabidopsis* and rice, two species without S-RNase-based SI. Subsequently, phylogenetic analysis of plant Skp1-like proteins show that all SSK1-like proteins from species employing S-RNase-based SI, along with LSK1, cluster together to form a monophyletic clade, diverged from well-characterized Skp1-like proteins, such as ASK1 (Fig. 3). LSK1 is a pollen-specific Skp1-like protein containing a 2-residue C-terminal tail as found in SSK proteins, identified in *Lilium longiflorum* employing GSI of an unknown molecular mechanism (Chang et al. 2009). These phylogenetic relationships imply that SSK proteins may also have a single origin, consistent with the evolution of S-RNases (Igic and Kohn 2001; Steinbachs and Holsinger 2002). In addition, the functional conservation of SSK proteins is also demonstrated by cross-binding assays between *Pyrus* and *Petunia*, in which PbSSK1 and PbSSK2 could interact with PhSLF and PhCUL1, and PhSSK1 could interact with PbSLF and PbCUL1, respectively (Fig. 6). Collectively, SSK proteins appear to represent a group of conserved pollen factors involved in *S*-RNase-based SI, and their cross-species binding ability with SLF and CUL1 further support that a conserved molecular and biochemical mechanism may be shared among species of Solanaceae, Plantaginaceae and Rosaceae. Further studies on the SSK proteins found in apple, tomato and potato, such as their expression patterns and potential interactions with SLF and CUL1, are required to test this scenario.

Possible roles of the putative SCF^{SLF} complex in S-RNase-based SI

To date, two alternative models have been proposed to explain the biochemical mechanism of S-RNase-base SI in Solanaceae and Plantaginaceae. The degradation model involves a putative canonical SCF^{SLF} complex, composed of SSK, SLF and CUL1, or a non-canonical SCF^{SLF} complex containing SBP1, SLF and CUL1, which are proposed to specifically target non-self S-RNase for ubiquitination and subsequent degradation through the ubiquitin proteasome pathway leading to non-self pollen acceptance, while leave self S-RNase intact to exert its cytotoxic activity resulting in self-pollen rejection (Zhang et al. 2009; Meng et al. 2011). The compartmentalization model is suggested in *Nicotiana*, in which non-self pollen acceptance is due to a stable sequestration of non-self S-RNase in vacuolar compartments of pollen tube rather than degradation (Goldraij et al. 2006). Nevertheless, the identification of a putative SCF^{SLF} (SSK/CUL1/SLF) complex in *Pyrus* in this study, together with the recently identified SCF^{SFB} complex in *Prunus* (Matsumoto et al. 2012), imply that such a degradation model may also work in the species from Rosaceae. In consistent, the ability of SLF binding of S-RNases observed in *Pyrus* suggests the possibility that S-RNases are the substrates of the putative SCF^{SLF} complex, and the conserved role of SSK in the putative SCF^{SLF} complex between *Pyrus* and *Petunia* implies the conservation of the molecular and biochemical mechanism of S-RNase-based SI among Solanaceae, Plantaginaceae and Rosaceae, although genetic evidence has suggested that *Prunus* seems to adopt a different SI strategy in which SLF/SFB is assumed to serve as a protector to avoid self S-RNase from detoxification rather than a destroyer as observed in the species from Solanaceae, Plantaginaceae and the tribe Pyreae of Rosaceae (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006a; Tsukamoto et al. 2006; Vilanova et al. 2006). Further studies focusing on the interaction affinity analysis of SLF with self/non-self S-RNases, the ubiquitination analysis of S-RNase after compatible and self-incompatible pollinations, and the genetic characterization of SSK proteins, would shed light on the detailed mechanism of the SCF^{SLF} complexes in S-RNase-based SI.

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