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The Skp1-like protein SSK1 is required for cross-pollen compatibility in *S*-RNase-based self-incompatibility

Lan Zhao[1,2,†], Jian Huang[1,†], Zhonghua Zhao[1,2,†], Qun Li[1], Thomas L. Sims[3] and Yongbiao Xue[1,*]

[1]*Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences and National Center for Plant Gene Research, Beijing 100190, China,*

[2]*Graduate University of Chinese Academy of Sciences, Beijing 100049, China, and*

[3]*Department of Biological Sciences and Plant Molecular Biology Center, Northern Illinois University, DeKalb, IL 60115 – 2861, USA*

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[*]For correspondence (fax +86 10 6253 7814; e-mail ybxue@genetics.ac.cn).

[†]These authors contributed equally to this work.

SUMMARY

The self-incompatibility (SI) response occurs widely in flowering plants as a means of preventing self-fertilization. In these self/non-self discrimination systems, plant pistils reject self or genetically related pollen. In the Solanaceae, Plantaginaceae and Rosaceae, pistil-secreted *S*-RNases enter the pollen tube and function as cytotoxins to specifically arrest self-pollen tube growth. Recent studies have revealed that the *S*-locus F-box (SLF) protein controls the pollen expression of SI in these families. However, the precise role of SLF remains largely unknown. Here we report that PhSSK1 (*Petunia hybrida* SLF-interacting Skp1-like1), an equivalent of AhSSK1 of *Antirrhinum hispanicum*, is expressed specifically in pollen and acts as an adaptor in an SCF(Skp1-Cullin1-F-box)^{SLF} complex, indicating that this pollen-specific SSK1-SLF interaction occurs in both *Petunia* and *Antirrhinum*, two species from the Solanaceae and Plantaginaceae, respectively. Substantial reduction of PhSSK1 in pollen reduced cross-pollen compatibility (CPC) in the *S*-RNase-based SI response, suggesting that the pollen *S* determinant contributes to inhibiting rather than protecting the *S*-RNase activity, at least in solanaceous plants. Furthermore, our results provide an example that a specific Skp1-like protein other than the known conserved ones can be recruited into a canonical SCF complex as an adaptor.

Keywords: SSK1, *S*-locus F-box protein, *S-RNase*, self-incompatibility, cross-pollen compatibility, *Petunia*.

INTRODUCTION

Self-incompatibility (SI) occurs widely in flowering plants as a means of preventing inbreeding and promoting out-crossing (de Nettancourt, 2001; Franklin-Tong, 2008). In the SI response, self or genetically related pollen is rejected by the pistil while non-self pollen is accepted for fertilization. These outcomes are referred to as self-pollen incompatibility (SPI) and cross-pollen compatibility (CPC), respectively. Among several different types of SI systems, the Solanaceae, Plantaginaceae and Rosaceae appear to employ a similar SI mechanism (Takayama and Isogai, 2005). In these families, the SI specificity is determined by a ribonuclease protein (*S*-RNase) expressed in the pistil and an F-box protein (SLF or SFB) in pollen (Lai *et al.*, 2002; Entani *et al.*, 2003; Ushijima *et al.*, 2003; Sijacic *et al.*, 2004). This type of SI is often referred to as *S*-RNase-based SI (Kao and Tsukamoto, 2004; McClure, 2004; Franklin-Tong, 2008; Zhang *et al.*, 2009).

Previous studies have demonstrated that the pistil *S* gene, the *S*-RNases are secreted by the pistil, taken up by growing pollen tubes in an *S*-haplotype independent way, and function as cytotoxins to arrest self-pollen tube growth (Luu *et al.*, 2000; Goldraij *et al.*, 2006). While on the other hand, to be an F-box protein (FBP), the pollen *S* determinant, SLF/SFB was supposed to form an SCF (Skp1/Cullin1/F-box) complex, functioning as an E3 ubiquitin ligase in the ubiquitination-26S proteasome system (UPS). Then, how does SLF/SFB work together with *S*-RNase leading to the inhibition of the growth of self pollen tubes but not non-self pollen tubes? To explain this, the simple inhibitor model has been proposed. In this model, the pollen *S* determinant is assumed to inhibit all *S*-RNases except that for a corresponding *S*-haplotype (Dodds *et al.*, 1996; Kao and McCubbin, 1996; Luu *et al.*, 2000). This model is supported

by a well known phenomenon termed competitive interaction and by gain-of-function transformation experiments (de Nettancourt, 2001; Franklin-Tong, 2008; Qiao *et al.*, 2004a; Sijacic *et al.*, 2004; Tsukamoto *et al.*, 2005). However, there is no loss-of-function evidence to support this model. Based on this model, if the function of the pollen *S* was missing, the pollen would lose its ability to inhibit the cytotoxins of all *S*-haplotype *S*-RNases. Nevertheless, surprisingly, the pollen *S*, *SFB* deletion or alteration was observed in self-compatible *Prunus*, suggesting that expression of pollen *S* in *Prunus* may be necessary for *S*-RNases to reject self pollen (Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005). Also, heteroallelic pollen with two different *SFB* genes produced by tetraploid *Prunus*, a species in the Rosaceae, is still self-incompatible and does not show competitive interaction (Hauck *et al.*, 2006). Therefore, an alternative model, the general inhibitor model was proposed based on the studies in Rosaceae. In the general inhibitor model, the function of the pollen *S* is assumed to protect self *S*-RNases from being inhibited by a general inhibitor of all *S*-RNases without any *S*-haplotype specificity (Luu *et al.*, 2001). Together, it appears that the Solanaceae, Plantaginaceae and Rosaceae could have different pollen rejection mechanisms despite sharing *S*-RNase as the pistil component. However, understanding the real function of the pollen *S* in the *S*-RNase-based SI responses depends on uncovering the biochemical mechanism of the SLF action. Most F-box proteins serve as components of a multi-subunit E3 ubiquitin ligase, named SCF complex, composed of Skp1, Cullin1, the F-box protein and Rbx1 (Schwechheimer and Calderon Villalobos, 2004; Petroski and Deshaies, 2005). Therefore, as an F-box protein (FBP), SLF was proposed to be the substrate receptor of an SCF ubiquitin ligase that might inhibit non-self *S*-RNases through the UPS (Lai *et al.*, 2002; Qiao *et al.*, 2004b; Hua and Kao, 2006, 2008; Zhang *et al.*, 2009). Nevertheless, this scenario is largely undefined. Intriguingly, an unorthodox E3-like complex containing SLF, Cullin1 and a RING-HC protein SBP1 was identified in *P. inflata* (Hua and Kao, 2006). Combined with the fact that most SCF ubiquitin ligases contain the conserved Skp1, it would be interesting to study the SLF-interacting partners and their role in SI responses.

Recently, another divergence of proposed models has arisen for the Solanaceae-type SI responses. These models proposed different mechanisms for how the pollen *S* products restrict the cytotoxity of the self *S*-RNases differently in the Solanaceae. They are referred to as the *S*-RNase degradation model and the compartmentalization model, respectively. In the degradation model, the SLF is suggested to function in an SCF^{SLF} ubiquitin ligase and might inhibit non-self *S*-RNases through the UPS, since the ubiquitination of non-self *S*-RNases was observed both *in vitro* and *in vivo* (Qiao *et al.*, 2004b; Sijacic *et al.*, 2004; Hua and Kao, 2006, 2008; Sims, 2007; Zhang *et al.*, 2009). However, in the compartmentalization model, after uptake of self and non-self *S*-RNases by pollen tubes, self *S*-RNase enters the cytoplasm of the pollen tube to function as cytotoxin, which is similar to the *S*-RNase degradation model, while non-self *S*-RNases appear to be compartmentalized in pollen vacuoles instead of being degraded in the CPC response (Goldraij *et al.*, 2006). Thus, it is essential to investigate whether the SLF functions in an SCF^{SLF} complex with non-self *S*-RNases as substrate. In this context, functional dissection of SLF-interacting partners would be critical for revealing the biochemical mechanisms of *S*-RNase-based SI.

Previously, we identified a novel Skp1-like protein, SLF-interacting Skp1-like1 (SSK1) in *Antirrhinum* and found that it could act as a specific adaptor of the SCF^{SLF} complex (Huang *et al.*, 2006). Here we report that SSK1 is also conserved in *Petunia hybrida* and is required for the CPC. Consistent with genetic studies (Golz *et al.*, 2001; Xue *et al.*, 2009), our results show that the pollen *S* determinant inhibits the activity of non-self *S*-RNases in the CPC response in the Solanaceae. Furthermore, our results provide new insights into the biochemical mechanism of *S*-RNase-based SI.

RESULTS

Molecular identification of PhSSK1 from *P. hybrida*

Previously, we had identified *AhSSK1* through yeast two-hybrid screening against a pollen cDNA library in *Antirrhinum* (Huang *et al.*, 2006). Pull-down assays suggested that this novel Skp1-like protein could be the specific adaptor for the pollen *S* AhSLF to form an SCF^{SLF} complex functioning in SI responses. Unfortunately, we could not dissect the role of *AhSSK1 in vivo* through a reverse genetic approach in *Antirrhinum hispanicum*, which can not be routinely transformed. Thus, we attempted to identify an AhSSK1 equivalent in *P. hybrida*, a solanaceous plant that is transformable and shares a similar SI mechanism to *A. hispanicum*. Among three Skp1-like proteins we identified in pollen by homology-based cloning and annotation searches in SGN (SOL Genomics Network, http://solgenomics.net) (Mueller *et al.*, 2005) (data not shown), one predicted protein shared the highest amino acid sequence similarity with AhSSK1 of 48.3% (Figure 1a). This protein was named PhSSK1 (*Petunia hybrid* SLF-interacting Skp1-like1, see below).

PhSSK1 encodes a predicted polypeptide of 179 amino acid residues. To examine whether PhSSK1 could interact with SLFs as AhSSK1 did, *PhSSK1* was cloned into *pGADT7* vector as a prey for yeast two-hybrid assays with several SLFs of *A. hispanicum* and *P. hybrida* (Figure S1), respectively. As observed for AhSSK1, PhSSK1 also showed strong interactions with both AhSLF-S_2 and AhSLF-S_5 (Figure 1b). In addition, both AhSSK1 and PhSSK1 showed strong interaction with PhSLF-S_V, indicating that the SSK1-SLF interaction is conserved in two species sharing the similar *S*-RNase-based SI (Figure 1b). In addition, the SSK1-SLF interaction

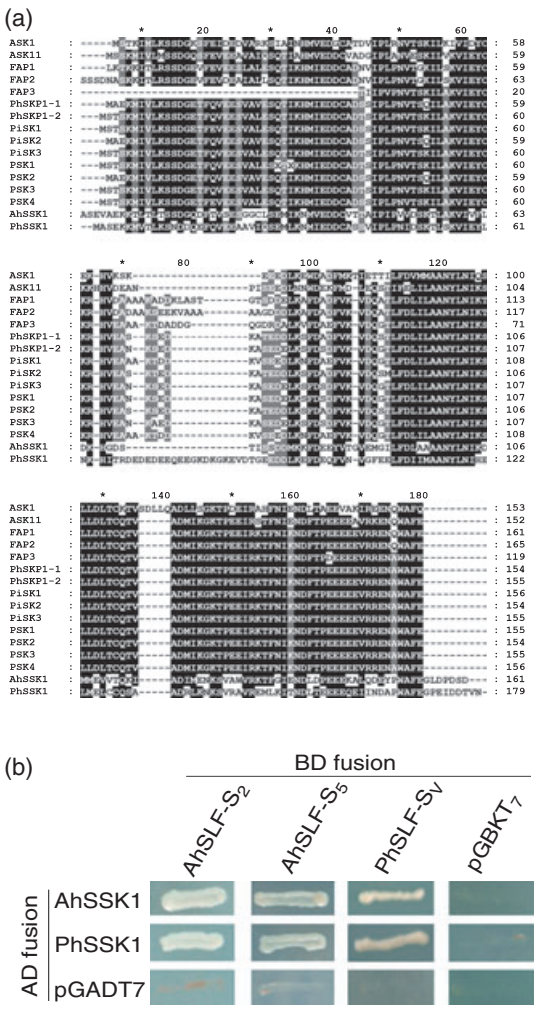

Figure 1. PhSSK1 is an ortholog of AhSSK1 and interacts with AhSLF-S_2/-S_5 and PhSLF-S_V.
(a) Amino acid sequence alignment of PhSSK1 and several typical plant Skp1–1ike proteins. ASKs and FAPs (FAP1, CAA75117; FAP2, CAA75118; FAP3, CAA75119) are from *Arabidopsis* and *Antirrhinum*, respectively. PhSKPs, PiSKs and PSKs are from *Petunia*. Residues of over 80% similarity are shaded in black, those of 60–80% similarity in dark gray and 30–60% similarity in light gray. Numbers show the positions of residues.
(b) Yeast two-hybrid assays of PhSSK1 with AhSLF-S_2/-S_5 and PhSLF-S_V. The empty vectors pGBKT7 and pGADT7 are negative controls.

was highly specific (Table S1); those housekeeping Skp1-like proteins that could be associated with many FBPs did not bind SLF and the SLF paralogs showed no apparent interaction with the SSK1 proteins (Zhou *et al.*, 2003; Huang *et al.*, 2006). Based on these observations, we conclude that PhSSK1 is an equivalent of AhSSK1 and is therefore likely to be involved in the *S*-RNase-based SI response.

SSK1 is a pollen-specific but not an *S*-haplotype-specific gene

To further characterize *PhSSK1*, we carried out Southern blots and expression analyses. Only one band was observed in each of the three wild-type *P. hybrida* lines of different *S*-haplotypes in Southern blots, suggesting that *PhSSK1*, similar to AhSSK1, is not an *S*-haplotype-specific gene (Figure 2a) (Huang *et al.*, 2006). Also as observed for *AhSSK1*, *PhSSK1* was exclusively expressed in pollen, as indicated by RT-PCR, quantitative RT-PCR and western blot results (Figure 2b–d). Furthermore, western blots showed that PhSSK1 begins to accumulate at stage III and reaches the peak in mature pollen (PS VI) during pollen development (Figure S2). Taken together, *SSK1* is a pollen-specific but not an *S*-haplotype-specific gene and both its transcripts and protein accumulate in mature pollen, suggesting that it could be required for pollen development and/or pollination processes.

SSK1 represents a novel class of Skp1-like proteins

AhSSK1 and PhSSK1 share ~40% amino acid identities with other Skp-1 like proteins (Figure 1a), and both were also predicted to have a similar secondary structure as human Skp1 protein (Figure S3) (Schulman *et al.*, 2000). However, it is noteworthy that both PhSSK1 and AhSSK1 have a unique C-terminus, composed of a 7–9 amino acid residue tail of disordered coil, following the conventional terminal residues 'WAFE' found in most plant Skp1 homologs (Figure 1a) (Gagne *et al.*, 2002; Risseeuw *et al.*, 2003; Huang *et al.*, 2006). Interestingly, no interaction between any SLF and the truncated SSK1 without the special C terminal tail was observed in yeast, suggesting that the disordered coil tail is important for its interaction with SLFs (Table S1). Furthermore, many residues within the backbone of SSK1 are different from those in the conventional Skp1 proteins (Figure 1a). These distinct features suggest that SSK1 represents a new type of Skp1-like protein. Also, AhSSK1 and PhSSK1 are the closest neighbors within a single clade of a phylogenetic tree of Skp1-like proteins (Figure S4), diverged from several housekeeping plant Skp1-like proteins, such as ASK1 and FAP1 (Ingram *et al.*, 1997; Zhao *et al.*, 2003). Notably, we could not identify any SSK1-like gene in *Arabidopsis* and rice genomes. Considering their pollen-specific expression and SLF-specific interaction, it might be proposed that SSK1 could be an SLF-related Skp1-like protein functions as a specific reproductive protein mainly in the *S*-RNase-based SI response.

Substantial down-regulation of PhSSK1 leads to a reduced fertility of cross-compatible pollen

To examine the role of *PhSSK1 in vivo*, a transgenic construct (*pBI101-LAT52-PhSSK1-RNAi*; Figure S5) was introduced into a self-incompatible *P. hybrida* of $S_{3L}S_{3L}$ genotype to knockdown the *PhSSK1* expression in pollen. In total, seven T_0 transgenic lines were identified by Southern blot (Figure 3). Among these, line *B* carried five transgene copies, *J2*, *K4* and *H6* four copies, *J4* three copies and *E1* and *M8* a single copy. Expression analysis by quantitative RT-PCR

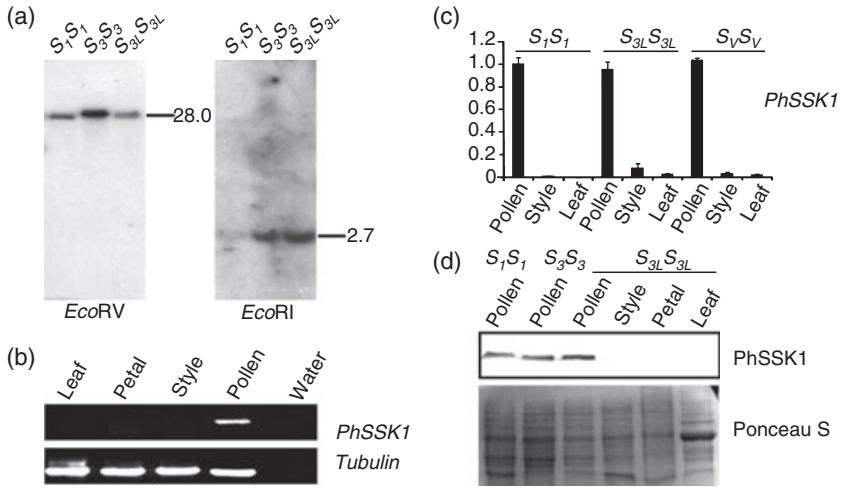

Figure 2. *PhSSK1* is a pollen-specific but not *S*-haplotype-specific gene in *Petunia hybrida*.
(a) Southern blot analysis of three *S* genotypes of self-incompatible *P. hybrida* (S_1S_1, S_3S_3 and $S_{3L}S_{3L}$). The genomic DNA was separately digested by *Eco*RV or *Eco*RI and probed by the full length (left) and partial (1–789 bp) (right) genomic DNA of *PhSSK1*, respectively. The numbers indicate fragment sizes in kilobase pairs.
(b–d). Expression pattern analyses of PhSSK1. The RNA and proteins of PhSSK1 in four tissues of different *S* haplotypes *P. hybrida* were examined by RT-PCR (b), quantitative RT-PCR (c) and western blot (d), respectively. *Tubulin* and gel staining by Ponceau S were loading controls of RT-PCR and western blot, respectively.

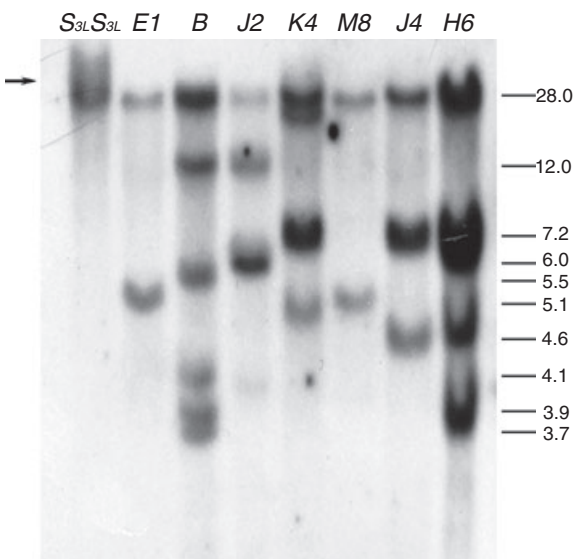

Figure 3. Identification of the T_0 *PhSSK1-RNAi* transgenic lines.
Southern blot analysis of seven T_0 transgenic lines. The numbers indicate fragment sizes in kilobase pairs. The arrow indicates the endogenous *PhSSK1*.

and western blots showed that *PhSSK1* mRNA and its protein were significantly reduced in the transgenic lines, especially in those lines carrying multiple trangene copies, such as *B*, *J2*, *K4* and *H6*, indicating that the reduction in *PhSSK1* mRNA expression was likely correlated with the transgene copy number (Figure S6). Moreover, the expression of the control gene *PhSKP1-2*, an *Skp1*-like gene, was not altered in the transgenic lines (Figure S6a), suggesting the RNAi effect was specific on *PhSSK1*. Thus, we obtained seven T_0 lines with variable reductions of PhSSK1 in pollen for subsequent functional analyses.

To examine whether any abnormal phenotype was caused by the reduction of *PhSSK1*, a general examination of the whole plant and pollen development as well as pollination behaviors of the T_0 lines was performed. The results showed that neither obvious morphological alteration of the whole plant nor abnormal pollination behavior of the style of these transgenic lines was observed (data not shown). Similarly, the self-pollination behavior of the pollen of these transgenic lines was not affected because they still remained self-incompatible. However, in cross-pollination tests, when the pollen grains of these transgenic plants were used to pollinate any other self-incompatible line (S_1S_1 or S_VS_V), fewer or even no seeds were produced, especially in those transgenic lines with a significant reduction in *PhSSK1* expression, such as *B* (Table S2), suggesting that a reduced fertility of cross-compatible pollen occurred in these transgenic lines.

To investigate whether decreased *PhSSK1* expression and the reduced fertility of cross-compatible pollen in the T_0 lines were caused by heritable changes rather than other factors like tissue culture effects, we generated T_1 progeny for several T_0 transgenic lines. Because *PhSSK1* expression in pollen was decreased to a greater degree and severe cross-pollination infertility was observed in those T_0 lines with multiple transgene copies, we mainly focused on the progeny derived from three T_0 lines, *B*, *K4* and *J2*. The T_1 lines were generated by using the S_VS_V wild-type plant as pollen donor to cross with the T_0 lines. Thus, the T_1 progeny was of $S_{3L}S_V$ genotype. A Southern blot of seven T_1 lines derived from the *B* (*VB* lines) showed that five plants carried the same number of the transgene copies as the maternal line *B* and two lines (*VB4* and *VB5*) carried three or two copies respectively, showing a segregation of the transgenes (Figure 4a), suggesting that the transgenes in the line *B* were located in two unlinked loci, one containing two copies and the other three. Similarly, three out of five T_1 lines from *K4* (*VK* lines) and nine out of 12 T_1 lines from *J2* (*VJ* lines) carried the same number of the transgene copies as *K4* and *J2*, respectively, and other progeny of *K4* and *J2* carried no

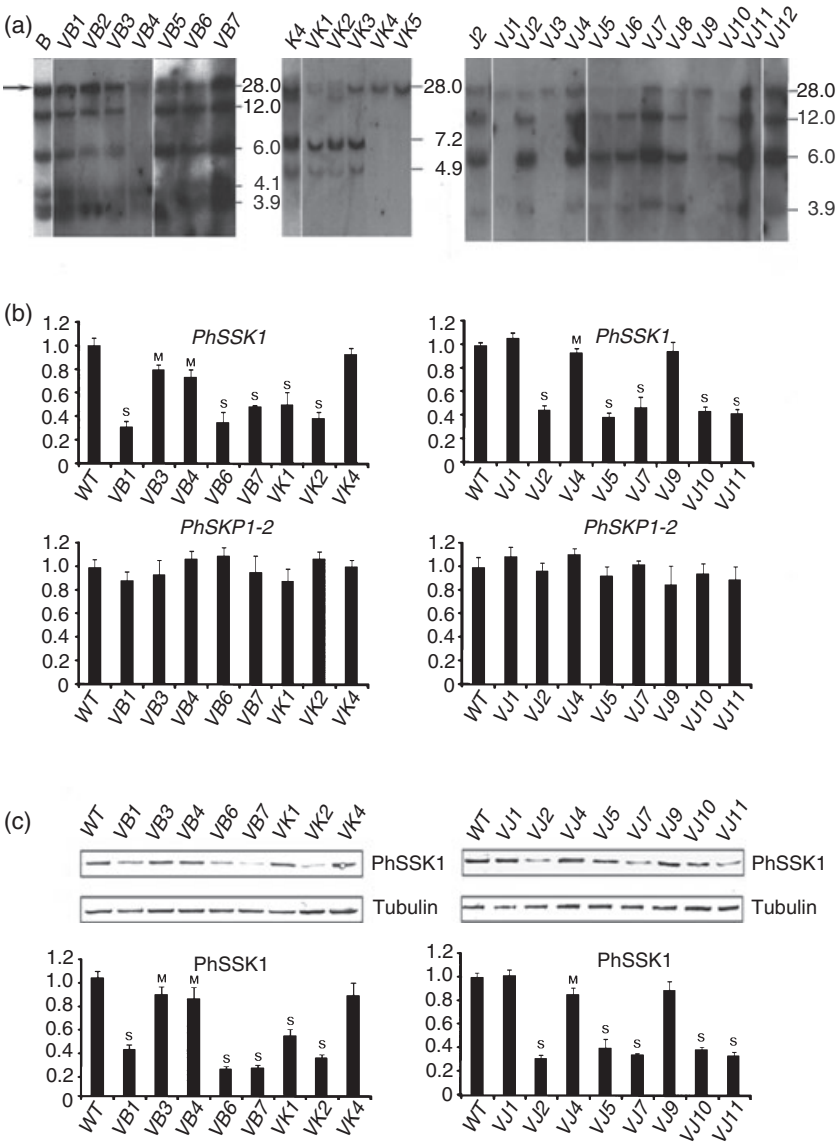

Figure 4. Molecular analyses of T_1 progeny of *B*, *K4* and *J2* crossed with S_VS_V as pollen donor.
(a) Southern analysis T_1 progeny of *B*, *K4* and *J2* crossed with S_VS_V as pollen donor. The genomic DNA was digested by *Eco*RV, and then probed by the full length genomic DNA of *PhSSK1*. The number indicates the fragment size in kilobase pairs and the arrow indicates the endogenous *PhSSK1*.
(b) Quantitative RT-PCR analysis of the expression of *PhSSK1* in the pollen of sixteen T_1 progeny with or without T-DNA. *PhSKP1-2* was a negative control.
(c) Western blot analysis of PhSSK1 expression in sixteen T_1 progeny. The PhSSK1 protein level of the T_1 lines was examined by western blot (top panel). Tubulin was a loading control. The relative protein amounts in these lines compared with wild-type pollen were calculated by the Quantity One (Bio-Rad) (bottom panel). The Marks 'S' and 'M' in B and C indicated lines of an over 40% reduction of PhSSK1 and lines of a < 20% reduction, respectively. The T_1 lines unmarked were those without T-DNA insertion lines.

transgene copy (Figure 4a), indicating that the transgenes in the T_0 lines *K4* and *J2* appeared to be linked closely, likely representing a single insertion locus.

To further examine whether the RNAi effects were heritable with the transgenes, we analyzed the expression of *PhSSK1* in pollen of the T_1 lines. Because all of the T_1 lines carrying the T-DNA copies were heterozygous for the transgenes, we reasoned that 50% of the pollen grains of the *K4* and *J2* progeny and some *B* progeny (*VB4* and *VB5*), and three-fourths of the pollen grains of the other five *B* progeny contained the transgenes. FiveT_1 progeny derived from *B*, three from *K4* and eight from *J2*, were then randomly chosen for further molecular analyses. Both quantitative RT-PCR and western blots consistently showed a substantial decrease of the *PhSSK1* mRNA and protein in the pollen grains of *VB1*, *VB6*, *VB7*, *VK1*, *VK2*, *VJ2*, *VJ5*, *VJ7*, *VJ10* and *VJ11*, with a reduction of ~40% (marked with 'S' for strong), and a moderate decrease in *VB3*, *VB4* and *VJ4*, with a reduction of ~20% (marked with 'M' for moderate), compared with that in the pollen grains of wild-type and the lines *VJ1* and *VJ9* without the transgenes (Figure 4b,c). These results showed that the RNAi transgene copies specifically reduced the expression of *PhSSK1* in pollen of the transgenic lines, suggesting that the RNAi effects were stably transmitted to T_1 progeny with the transgene copies.

To further examine the reduced fertility of the cross pollen observed in T_0 lines was correlated with down-regulation of *PhSSK1* in T_1 progeny, we performed pollination assays using the pollen grains of T_1 progeny with defined transgenes and *PhSSK1* knockdown. All of the T_1 transgenic lines tested showed normal self-incompatibility responses after self-pollination. However, when the pollen of seven T_1

progeny marked with 'S', with >40% reduction of the *PhSSK1* transcripts (see Figure 4b,c), pollinated the wild-type $S_{3L}S_{3L}$, fewer seeds were produced (<200), compared with the T_1 progeny marked with 'M', with <20% *PhSSK1* reduction (~250) (see Figure 4b,c), or wild-type $S_{3L}S_V$ (~400) (Table 1), confirming that the reduced fertility of cross-compatible pollen caused by the reduction of *PhSSK1* was inheritable.

Reduced fertility of cross pollen with SSK1 knockdown is due to a specific loss of cross-pollen compatibility

To examine whether the reduced fertility of compatible pollen was due to a defect in pollen development and/or aberrance of the transgenic pollen in the SI response, we then used the transgenic pollen grains from 10 T_1 lines with significant *PhSSK1* knockdown to pollinate a self-compatible S_OS_O line, where the self-compatibility was most likely due to a defective style SI function (Figure S7). In all pollinations with S_OS_O, whether self- or cross-pollinations, the number of seeds produced (~70) was less than that of cross-pollinations with the styles of SI wild-type lines (which was generally >100 seeds; the numbers of seed set could be different between wild-type lines). The pollination results showed that the sizes of the fruits obtained by the transgenic lines were similar to those using wild-type as pollen donor (Table 2). The segregation ratios of the *PhSSK1-RNAi* transgenes, and of the *S*-locus, in the T_2 progeny described above, were also examined. Based on the transgenic locus numbers of T_1 lines (*VB*s: one or two loci; *VJ*s and *VK*s: one locus), three-quarters of the T_2 progeny from *VB1*, *VB3* and *VB6* and half of the T_2 progeny from the other seven lines were expected to carry the *PhSSK1-RNAi* copies, if the knockdown of *PhSSK1*did not affect the normal pollen developmental processes. Otherwise, there should be a distortion of the transgene segregation ratios in the T_2 population. All 10 T_2 populations showed normal segregation ratios of both the *PhSSK1-RNAi* transgenes (either 1:1 or 3:1, respectively; chi-squared test, $P > 0.05$) and the *S*-locus (Table 2 and Figure S9), regardless of whether the pollen used was from T_1 lines with strong or mild reduction of *PhSSK1*, suggesting that the pollen with *PhSSK1* knockdown can grow through the *SoSo* styles normally and therefore succeed in fertilization, as wild-type pollen does in outcrossing. Taken together, these results showed that the reduced fertility of cross-compatible pollen was not caused by an adverse effect of decreased *PhSSK1* on pollen development, indicating that the CPC in the SI response might be affected in the transgenic lines.

Substantially down-regulated PhSSK1 reduced cross pollen compatibility (CPC) in the SI response

To confirm that the effect of the reduced PhSSK1 expression on CPC was related to the SI responses, we examined the transgene segregation ratios of the T_2 population as described in Table 1. Both the segregation ratios of the T-DNA and the *S*-locus were examined. Based on the transgene locus numbers of T_1 transgenic lines, we reasoned that the expected segregation ratio of T-DNA would be 3:1 or 1:1 respectively, if the reduction in *PhSSK1* expression did not affect the CPC. However, only 12/24 of *VB1* T_2 progeny, 11/24 of *VB6*, 5/24 of *VK1*, 3/24 of *VK2*, none of *VJ5*, 5/24 of *VJ10* and 1/24 of *VJ11* carried the T-DNA (Table 3 and Figure S10). Chi-squared tests showed that the segregation ratio distortion was significant ($P < 0.01$) in the T_2 population derived from these seven T_1 plants with a strong reduction of *PhSSK1*, suggesting that the reduced fertility of cross-pollen was due to a specific adverse effect of the *PhSSK1-RNAi* on CPC. We did not, however, find significant distortions in the T_2 progeny of *VB3*, *VB4* and *VJ4* (Table 3 and Figure S10), in which only moderate reduction of *PhSSK1* expression occurred (see Figure 4). Taken together, these results indicated that pollen with a greater reduction of *PhSSK1* expression (over 40%), was not transmitted normally due to the loss of CPC in *S*-RNase-based SI.

PhSSK1 specifically bridges PhSLF to Cullin1 to form an SCF complex

We next tried to examine how SSK1 functions in CPC. Pull-down assay showed that N-terminal regions of four PhSLFs containing the F-box domain could bound with PhSSK1 (Figure 5a), further confirming the SSK1-SLF interaction. As in a canonical SCF complex, the Skp1 protein is usually

Table 1 Pollination analyses of T_1 lines

	T_1[a]										
		$S_{3L}S_V$	*VB1* (S)	*VB3* (M)	*VB4* (M)	*VB6* (S)	*VK1* (S)	*VK2* (S)	*VJ4* (M)	*VJ5* (S)	*VJ10* (S)	*VJ11* (S)
$S_{3L}S_{3L}$	Seed set[b]	5 (5)	5 (5)	6 (6)	5 (4)	7 (7)	6 (4)	5 (5)	6 (5)	8 (6)	9 (6)	5 (5)
	Avg. seeds[c]	462 ± 47	156 ± 54	261 ± 23	225 ± 37	244 ± 16	190 ± 39	215 ± 45	254 ± 43	197 ± 39	60 ± 36	191 ± 23

[a]All T_1 lines were used to pollinate $S_{3L}S_{3L}$. The crosses between $S_{3L}S_{3L}$ and $S_{3L}S_V$ were compatible when the $S_{3L}S_V$ plant was pollen donor.
[b]The number of total pollinations (the number of the pollinations setting fruits).
[c]The number of average seeds, if pollination resulted in seed set.
Lines marked with (S) and (M) correspond to the lines with a strong and a mild reduction of *PhSSK1* (consistent with 'S' and 'M' in Figure 4), respectively.

Table 2 Pollination and T-DNA segregation analyses of T_1 lines shown in Table 1

Crosses[a]	Seed set[b]	Avg. seeds	Number of progeny[c]	Progeny	Expected segregation ratio[d]	Observed segregation ratio	χ^2 test[e] $\chi^2_{0.01} = 6.635$ $\chi^2_{0.05} = 3.841$
S_OS_OX$S_{3L}S_V$	5 (5)	63 ± 22	nt	nt	nt	nt	nt
S_OS_O X*VB1* (S)	7 (4)	57 ± 21	24	N_{T+}:N_{T-}	3:1	16:8	1.11, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	11:13	0.17, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	3:1	8:3	0.03, $P > 0.05$
				N_{T+SV}:N_{T-SV}	3:1	8:5	1.26, $P > 0.05$
S_OS_O X*VB3* (M)	7 (7)	85 ± 23	24	N_{T+}:N_{T-}	3:1	15:9	2.00, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	14:10	0.67, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	3:1	8:6	2.38, $P > 0.05$
				N_{T+SV}:N_{T-SV}	3:1	7:3	0.13, $P > 0.05$
S_OS_OX*VB4* (M)	6 (4)	63 ± 22	24	N_{T+}:N_{T-}	1:1	11:13	0.17, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	12:12	0, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	1:1	7:5	0.33, $P > 0.05$
				N_{T+SV}:N_{T-SV}	1:1	4:8	1.33, $P > 0.05$
S_OS_O X*VB6* (S)	7 (5)	68 ± 30	24	N_{T+}:N_{T-}	3:1	17:7	0.11, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	13:11	0.17, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	3:1	9:4	0.23, $P > 0.05$
				N_{T+SV}:N_{T-SV}	3:1	8:3	0.03, $P > 0.05$
S_OS_O X*VK1* (S)	6 (6)	84 ± 17	24	N_{T+}:N_{T-}	1:1	10:14	0.67, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	11:13	0.17, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	1:1	6:5	0.09, $P > 0.05$
				N_{T+SV}:N_{T-SV}	1:1	4:9	1.92, $P > 0.05$
S_OS_O X*VK2* (S)	6 (4)	86 ± 26	24	N_{T+}:N_{T-}	1:1	11:13	0.17, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	12:12	0, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	1:1	5:7	0.33, $P > 0.05$
				N_{T+SV}:N_{T-SV}	1:1	6:6	0, $P > 0.05$
S_OS_O X*VJ4* (M)	5 (4)	79 ± 26	24	N_{T+}:N_{T-}	1:1	13:11	0.17, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	11:13	0.17, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	1:1	6:5	0.09, $P > 0.05$
				N_{T+SV}:N_{T-SV}	1:1	7:6	0.08, $P > 0.05$
S_OS_O X*VJ5* (S)	7 (5)	87 ± 34	24	N_{T+}:N_{T-}	1:1	13:11	0.17, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	13:11	0.17, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	1:1	9:4	1.92, $P > 0.05$
				N_{T+SV}:N_{T-SV}	1:1	4:7	0.82, $P > 0.05$
S_OS_O X*VJ10* (S)	6 (4)	79 ± 28	24	N_{T+}:N_{T-}	1:1	10:14	0.67, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	12:12	0, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	1:1	5:7	0.33, $P > 0.05$
				N_{T+SV}:N_{T-SV}	1:1	5:7	0.33, $P > 0.05$
S_OS_O X*VJ11* (S)	6 (6)	90 ± 27	24	N_{T+}:N_{T-}	1:1	12:12	0, $P > 0.05$
				N_{S3L}:N_{SV}	1:1	10:14	0.67, $P > 0.05$
				N_{T+S3L}:N_{T-S3L}	1:1	6:4	0.40, $P > 0.05$
				N_{T+SV}:N_{T-SV}	1:1	6:8	0.29, $P > 0.05$

[a]All T_1 lines were used to pollinate the S_OS_O. The crosses between S_OS_O and $S_{3L}S_V$ were compatible.
[b]The number of overall pollinations (the number of the pollinations setting fruits).
[c]The number of T_2 plants of each line used for the segregation analyses.
[d]The T-DNA segregation ratio expected if the segregation is random. N_{T+}, the number of the plants with T-DNA; N_{T-}, the number of the plants without T-DNA; N_{S3L}, the number of plants with *S_{3L}-RNase;* N_{SV}, the number of plants with *S_V-RNase;* N_{T+S3L}, the number of plants with both T-DNA and *S_{3L}-RNase;* $N_{T\text{-}S3L}$, the number of plants with *S_{3L}-RNase* but without T-DNA; N_{T+SV}, the number of plants with both T-DNA and *S_V-RNase;* $N_{T\text{-}SV}$, the number of plants with *S_V-RNase* but without T-DNA.
[e]The chi-squared goodness-of-fit test of the segregation ratio observed; if the $\chi^2_{sample} > \chi^2_{0.01}$, the value of $P < 0.01$, which means there was a significant difference between the segregation ratio observed and that expected. Otherwise, if the $\chi^2_{sample} < \chi^2_{0.05}$, the value of $P > 0.05$, which means the segregation ratio observed is fit to the segregation ratio expected.
nt, indicates not tested.
Lines marked with (S) and (M) correspond to the lines with a strong and a mild reduction of *PhSSK1* (consistent with 'S' and 'M' in Figure 4), respectively.

recruited as the adaptor between the scaffold protein Cullin1 and an FBP, we then tested whether PhSSK1 could interact with Cullin1 in pollen. As Figure 5b shows, a Cullin1-like protein of the *Petunia* pollen was detected in a GST-PhSSK1 pull-down assay, which confirmed the physical interaction between SSK1 and Cullin1, indicating that SSK1-SLF-Cullin1

Table 3 T-DNA segregation analyses of T_1 lines

Crosses[a]	Numbers of plants[b]	Expected segregation ratio[c]	Observed segregation ratio	χ^2 test [d] $\chi^2_{0.01} = 6.635$, $\chi^2_{0.05} = 3.841$
$S_{3L}S_{3L}$X*VB1* (S)	24	3:1	12:12	8.00, $P < 0.01$
$S_{3L}S_{3L}$X*VB3* (M)	24	3:1	17:7	0.11, $P > 0.05$
$S_{3L}S_{3L}$X*VB4* (M)	24	1:1	13:11	0.17, $P > 0.05$
$S_{3L}S_{3L}$X*VB6* (S)	24	3:1	11:13	10.89, $P < 0.01$
$S_{3L}S_{3L}$X*VK1* (S)	24	1:1	5:19	8.17, $P < 0.01$
$S_{3L}S_{3L}$X*VK2* (S)	24	1:1	3:21	13.50, $P < 0.01$
$S_{3L}S_{3L}$X*VJ4* (M)	24	1:1	11:13	0.17, $P > 0.05$
$S_{3L}S_{3L}$X*VJ5* (S)	24	1:1	0:24	24.00, $P < 0.01$
$S_{3L}S_{3L}$X*VJ10* (S)	24	1:1	5:19	8.17, $P < 0.01$
$S_{3L}S_{3L}$X*VJ11* (S)	24	1:1	1:23	20.17, $P < 0.01$

[a]All T_1 lines were used to pollinate the wild-type $S_{3L}S_{3L}$. The crosses between $S_{3L}S_{3L}$ and $S_{3L}S_V$ were compatible when the $S_{3L}S_V$ plant was used as pollen donor.
[b]The number of T_2 progeny of each line used for T-DNA segregation analyses.
[c]T-DNA segregation ratios expected if the T-DNA segregation is random.
[d]The chi-square goodness-of-fit test of the segregation ratio observed; if the $\chi^2_{sample} > \chi^2_{0.01}$, the value of $P < 0.01$, which means that there is a significant difference between the segregation ratio observed and that expected. Otherwise, if the $\chi^2_{sample} < \chi^2_{0.05}$, the value of $P > 0.05$, which means the segregation ratio observed is fit to the segregation ratio expected.
Lines marked with (S) and (M) correspond to the lines with a strong and a mild reduction of *PhSSK1* (consistent with 'S' and 'M' in Figure 4), respectively.

could form a stable complex in *Petunia* pollen. A similar result was obtained when we used an *in vitro* transcription/translation system to express the PhSSK1 and PhCullin1 proteins and co-purified the PhSSK1–PhCullin1 complex (Figure 5c). Moreover, *S*-RNases were also shown to be in the pull-down proteins when both GST-PhSSK1 and MBP-PhSSK1 were used (Figure 5d). Together, our results showed that SSK1 serves as a specific adaptor to bridge SLF to Cullin1 proteins in *Petunia*, constituting an SCF complex targeting *S*-RNase, similar to that found in *Antirrhinum* (Huang *et al.*, 2006).

DISCUSSION

The SSK1, SLF and Cullin1 constitute a unique but canonical SCF^{SLF} complex

A canonical SCF complex is always composed of an FBP, Cullin1, a RING protein and Skp1, which usually acts as an adaptor bridging the FBP and Cullin1 (Petroski and Deshaies, 2005; Ho *et al.*, 2008). As an FBP, the pollen *S* determinant, SLF, was suggested to function in a putative SCF^{SLF} complex (Qiao *et al.*, 2004b; Hua and Kao, 2006, 2008; Xue *et al.*, 2009; Zhang *et al.*, 2009). Previous studies showed that SLF did not interact with conserved Skp1-like proteins, such as ASKs and PiSKPs (Zhou *et al.*, 2003; Hua and Kao, 2006; Huang *et al.*, 2006). Consistently, our results show that the *Petunia* homolog of *Antirrhinum* AhSSK1, PhSSK1, can specifically bridge PhSLF and PhCullin1 (Figure 5), suggesting that they together are capable of forming a typical SCF complex, similar to that found in *A. hispanicum* (Zheng *et al.*, 2002; Huang *et al.*, 2006). Nevertheless, the SCF^{SLF} complex is unique, as the adaptor, SSK1, is a novel Skp-1 like protein compared with those SKP1 homologs (Figures 1a and S4). So far, most studies on the Skp1-F-box interaction predominantly involve a few housekeeping Skp1-like proteins in each organism, such as ASK1/ASK2 in *Arabidopsis* and SKR-1 in *Caenorhabditis elegans*, leaving ~20 Skp1 homologs in a plant species as 'orphans'. Furthermore, only one Skp1 homolog has been identified in yeast or human. Thus, our results provide an example that a specific Skp1-like protein other than those conserved ones can be recruited into a canonical SCF complex as the adaptor. It is also notable that most currently documented plant SCF complexes play important roles in development and physiology, such as light and hormone signaling (Moon *et al.*, 2004; Smalle and Vierstra, 2004). Thus, the SCF^{SLF} may represent a new family of SCF complexes which recruit a pollen-specific Skp1-like protein as the adaptor and the SSK1 represents an Skp1-like protein that is reported to have a specific role in a defined biological pathway.

However, another complex containing SLF has recently been suggested by Hua and Kao (2006), who argued that the complex containing PiSLF is not a canonical SCF complex because they showed that PiSBP1, a RING-HC protein almost identical to *P. hybrida* SBP1 (Sims and Ordanic, 2001), also interacts with *S*-RNases, PiSLFs, PiCullin1-G and a ubiquitin-conjugating enzyme *in vitro*. In their proposed SCF^{SLF} complex, PiCullin1-G, SBP1 and SLF may be components of a novel E3 ubiquitin ligase complex, with PiSBP1 playing the roles of Skp1 and Rbx1 (Hua and Kao, 2006). Until now, there was no evidence for the relationship of the two different putative SCF^{SLF} complexes. However, recent evidence suggests that an FBP always needs an association

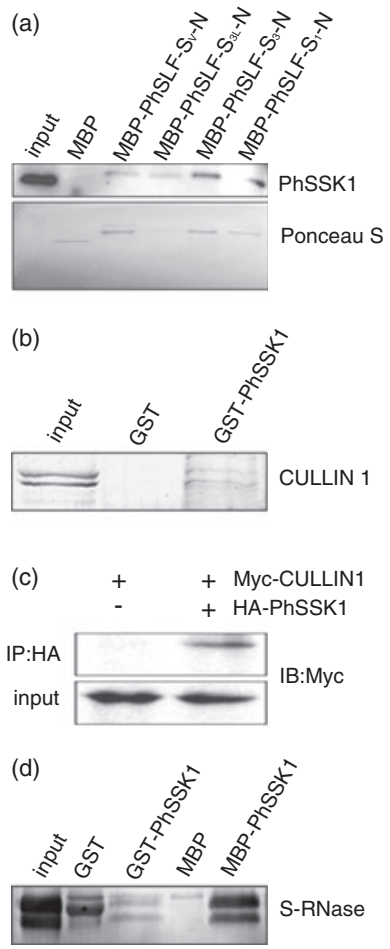

Figure 5. PhSSK1 is a specific adaptor that bridges SLF to Cullin 1.
(a) Pull-down assay of PhSSK1 with PhSLFs. The fusion proteins of MBP with the N-terminal region of four *S* haplotype SLFs (MBP-SLF-S_V/-S_{3L}/-S_1/-S_3-N) were used in a pull-down assay against pollen total protein. Bound protein was examined with the anti-PhSSK1 antisera. MBP was a negative control.
(b) Pull-down assay of PhSSK1 and Cullin 1. GST-PhSSK1 fusion protein was used in a pull-down assay against the pollen total protein. Bound protein was examined with the anti-AtCullin1 antibody. GST was a negative control.
(c) Immunoprecipitation assays of PhSSK1 and PhCullin1. PhSSK1 tagged with HA and PhCullin1 tagged with Myc were expressed *in vitro* transcription/translation system, respectively. Immunoprecipitation assays were performed using anti-HA antibody and immuno-purified complexes were examined with anti-Myc antibody. Three percent of the expression lysate was loaded as input.
(d) Pull-down assays of PhSSK1 and *S*-RNases. Mixed protein extracts of pollen and style were used for the pull-down assays with GST-PhSSK1 and MBP-PhSSK1, respectively. Bound protein was examined with the anti-PhS-RNase antibody. The asterisk denotes the GST protein that cross-reacts with the antiserum. GST and MBP were negative controls, respectively.

with Skp1 to fulfill its function (Kipreos and Pagano, 2000; Hermand, 2006; Ho *et al.*, 2008), indicating that if SBP1 is a component of the SCF^{SLF} complex, it might be possible that it forms the complex together with an Skpl-like molecule, but not replacing it. Given that SBP1 is not a tissue-specific protein (Sims and Ordanic, 2001; Hua and Kao, 2006), another possibility would be that SBP1 participates in more general biological processes, such as a downstream reaction of SI response.

The role of SSK1 in *S*-RNase-based SI responses

Although several factors including the pollen *S* and the style *S* determinants have been identified in several *S*-RNase-based SI species, their specific roles in CPC and SPI responses are not entirely clear. Among these factors, stylar HT-B and 120K proteins appear to be involved in the SPI response because their down-regulation led to breakdown of self-incompatibility (McClure *et al.*, 1999; O'Brien *et al.*, 2002; Hancock *et al.*, 2005; Puerta *et al.*, 2009). SBP1 appears to be a factor involved in the CPC response based on *in vitro* biochemical evidence showing that it is required for *S*-RNase ubiquitination and degradation (Sims and Ordanic, 2001; Sims, 2005; Hua and Kao, 2006). In this study, we have shown that a new Skp1-like protein, SSK1, is required for the CPC in *S*-RNase-based SI. First, pollen-specific SSK1 proteins specifically interact with SLF in both *Antirrhinum* and *Petunia* (Figures 1 and 5, and Table S1). Second, the substantial reduction of PhSSK1 reduced the CPC during the SI response (Tables 1–3). Third, the SSK1 proteins form a monophyletic group and appear not to be present in *Arabidopsis* and rice (Figure S4), which apparently lack the *S*-RNase-based SI system. Our findings show that SSK1 is an SI-related Skp1-like protein functioning in CPC signaling in the Solanaceae and Plantaginaceae.

Nevertheless, it is unclear that whether SSK1 is also involved in the SPI response and/or other unknown pathways. Because the PhSSK1-RNAi transgenic plants remained self-incompatible, it was impossible for us to investigate its role in the SPI reaction. Previous studies have shown that SLF interacts with both self- and non-self *S*-RNases with an apparently different affinity, i.e., a stronger interaction with non-self *S*-RNases than that with self *S*-RNases (Hua and Kao, 2006). It has been shown that whether FBPs function in an SCF-dependent or SCF-independent way, Skp1 is always recruited (Hermand, 2006; Ho *et al.*, 2008). Thus, it is possible that SSK1 as an SLF interacting Skp-1 like protein might also be involved in the SPI response. *In vivo* examination of SLF function by a loss-of-function approach could help to resolve these possibilities.

Furthermore, our results of PhSSK1 down regulation are consistent with the simple inhibitor model. Since SSK1 likely functions as the adaptor in the SCF^{SLF} complex, it may be proposed that any dysfunction of this SCF complex, such as a deletion of the *SLF* gene or the loss of a functional SSK1-SLF interface, will unleash the non-self *S*-RNases that would be inhibited in a normal cross-pollination. In other words, the pollen determinant acts to negatively regulate non-self *S*-RNases in the Solanaceae and most likely in the Plantaginaceae as well.

Possible biochemical mechanisms of *S*-RNase-based SI responses

Currently, two models have been proposed to explain the restriction mechanism of non-self *S*-RNase. The degradation model posits that as an FBP, the pollen *S* of SLF forms an E3 ligase SCF^{SLF} complex, which is capable of ubiquitinating non-self *S*-RNases with the polyubiquitinated *S*-RNases subsequently degraded by the 26S proteasome (Qiao *et al.*, 2004b; Sijacic *et al.*, 2004; Hua and Kao, 2006, 2008). Recently, an *S*-RNase compartmentalization model has been suggested in *Nicotiana* (Goldraij *et al.*, 2006). In this model, non-self *S*-RNases are compartmented in vacuoles of the pollen tubes rather than being degraded. Although the loss of the CPC by the reduced expression of PhSSK1 could not answer the question of the fate of the non-self *S*-RNases directly during compatible pollination, our findings imply that ubiquitination of non-self *S*-RNases could be indeed involved in the CPC. First, SSK1 together with SLF and Cullin1 form a canonical SCF^{SLF} complex. Second, *S*-RNases could be pulled-down *in vitro* by SSK1 from cross-pollinated pollen (Figure 5), indicating that the *S*-RNases were the substrates of the SCF^{SLF} complex. Nonetheless, it is known that ubiquitination can serve as a signal not only for 26S proteasome degradation but also for endocytic and vesicle-trafficking machineries (Chen and Sun, 2009). Further studies along these lines will generate a better understanding of self and non-self pollen recognition and their fates in *S*-RNase-based SI.

In conclusion, our results provide strong evidence that SSK serves as a novel adaptor for a canonical SCF^{SLF} complex and is required for cross-pollen compatibility in *S*-RNase-based SI, consistent with genetic studies showing that pollen *S* functions as an inhibitor of non-self *S*-RNases in the Solanaceae and Plantaginaceae. Nevertheless, a role of SSK1 in the incompatible response, if any, remains to be investigated.

EXPERIMENTAL PROCEDURES

Plant materials

Wild-type self-incompatible *P. hybrida* lines ($S_{3L}S_{3L}$, S_VS_V, S_1S_1 and S_3S_3) have been previously described (Clark *et al.*, 1990; Robbins *et al.*, 2000; Qiao *et al.*, 2004a). Because the *Petunia* line of S_3S_3 used in our current study was first described by Clark *et al.* (1990), we renamed the S_3S_3 genotype used in Qiao *et al.* (2004b) as $S_{3L}S_{3L}$ genotype to differentiate these two lines. Self-compatible *P. hybrida* line (S_OS_O) was isolated from a commercial stock (Taikeseed, http://www.taikeseed.com).

Leaf disk transformation of wild-type $S_{3L}S_{3L}$ line with *Agrobacterium* was used to generate transgenic plants as described previously (Lee *et al.*, 1994; Qiao *et al.*, 2004a).

Yeast two-hybrid assay

Yeast two-hybrid analysis was performed as previously described, with minor modifications (Huang *et al.*, 2006). The transformants streaked on SD/-Ade-His-Leu-Trp were allowed to grow for 10 more days at 18°C before they were photographed.

Molecular techniques

Genomic DNA isolation and Southern blot was performed as described previously (Qiao *et al.*, 2004a).

Total RNA was prepared as previously described (Lai *et al.*, 2002). The cDNA was produced using SuperScript reverse transcriptase (Invitrogen, http://www.invitrogen.com) and a poly-dT primer. Quantitative RT-PCR was performed using the SYBR Green PCR Master Mix and the ABI 7900 Sequence Detection System (ABI, http://www.appliedbiosystems.com) according to the manufacturer's protocol. Gene-specific primers listed in Table S3 were designed by using PRIMEREXPRESS 1.0 software (ABI). The 18S *rRNA* was used as an internal control to normalize all data.

Total pollen protein was prepared as previously described (Huang *et al.*, 2006) and then added with equivalent volume of 2× loading buffer (20% glycerol, 100 mM Tris-Cl (pH6.8), 0.04% bromophenol blue, 4% SDS and 4% 2-mercaptoethanol), boiled for 5 min. The samples were separated on SDS-polyacrylamide gels, and then transferred to PVDF membranes (GE healthcare, http://www.gehealthcare.com/cnzh) for western blot analyses. Anti-PhSSK1 antibody were prepared using purified GST-PhSSK1as antigens in rabbit, as described by Qiao *et al.* (2004a) and anti-tubulin monoclonal antibodies were from Sigma-Aldrich (http://www.sigmaaldrich.com). Western blots were treated with anti-PhSSK1 antibody (1:1000) or anti-tubulin (1:2000), respectively, and were detected using alkaline phosphatase-conjugated secondary antibodies and BCIP/NBT (Sigma-Aldrich). The relative protein amount of PhSSK1 was calculated by Quantity One (Bio-rad, http://www.bio-rad.com) using tubulin as reference protein.

Genotype identification assays of T_2 progeny were conducted by PCR using gene-specific primers listed in Table S3.

In-gel assay of RNase activity

Crude extracts of plant protein were prepared from the styles of open flowers as previously described (Huang *et al.*, 2006). The protein sample was prepared as described by McCubbin *et al.* (1997) and separated on a 12% SDS-polyacrylamide gel with 300 μg ml^{-1} of torula yeast tRNA (Sigma-Aldrich) added before casting. After electrophoresis, the gel was processed essentially as described (Brown and Ho, 1986). Then the gel was stained for RNA with 0.2% toluidine blue in the reaction buffer.

Pull-down assays

The N-terminus (1–273 bp) of *PhSLF* cDNA sequences were cloned into pMAL-c2x (NEB, http://www.neb.com) to generate *MBP-PhSLF-N* fusion constructs; the full length cDNA of PhSSK1 was cloned into *pGEX-4T-1* (GE Healthcare) and pMAL-c2x to generate the *GST-PhSSK1* and *MBP-PhSSK1* fusion constructs, respectively. MBP or GST fusion proteins were prepared as previously described (Huang *et al.*, 2006). Clarified lysates were incubated with amylose resin (NEB) or glutathione-Sepharose 4B beads (GE Healthcare) for 1 h at 25°C in PBS buffer. Beads were then washed four times with 10 bed volumes of PBS (10 mM maltose or 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0).

Anti-AtCullin1 antibody was described previously (Qiao *et al.*, 2004b). Anti-PhS-RNase antibody was prepared using purified GST-PhS_{3L}-RNase as antigens in rabbit, as described by Qiao *et al.* (2004a). The cDNA of *PhS-RNase-*S_O was cloned to pET28a nd the antigen of PhS-RNase-S_O prepared using purified His-PhS-RNase-S_O.

Crude extracts of plant protein were prepared from open flowers as previously described (Huang *et al.*, 2006). The cleared pollen

crude extracts were incubated with 20 μg purified MBP or GST fusion protein bound to 30 μl stacked amylose resin or glutathione-Sepharose 4B beads for 3 h at 4°C, respectively. After the beads were washed four times with the lysis buffer containing 0.1% Triton X-100, pull-down proteins were eluted with PBS elution buffer. The eluates were used for western blot analyses probed with anti-PhSSK1 or anti-AtCullin1 antibody. The similar procedure was applied for *S*-RNase detection in GST/MBP-PhSSK1 pull-down, except that the pollen and style extracts were combined in a ratio of 1:1 to generate crude protein extracts.

In vitro transcription/translation and immunoprecipitation

PhCullin1 was cloned from the pollen cDNA of *P. hybrida* by RT-PCR using gene-specific primers (Table S3) and cloned into the pGEM-T-easy vector (Promega, http://www.promega.com). For *in vitro* immunoprecipitation experiments, 1 μg of pGEM-PhCullin1 (Myc-tagged) and 1 μg of pGADT7 or pGADT7-PhSSK1 (HA-tagged) vectors were used in the TnT Quick Coupled Transcription/translation System (Promega) as specified by the manufacturer. *In vitro* translated proteins were mixed with 1 μg of anti-HA antibody (Sigma-Aldrich) and incubated with rotation at 4°C. After 2 h, 20 μl of EZ-view Red Protein G Affinity Gel (Sigma-Aldrich) were mixed with the lysate mixture and incubated at 4°C for further 2 h. After beads were washed in 0.5 ml of lysis buffer as described in the part of the pull-down assays three times, samples were resuspended in 30 μl of SDS-PAGE sample buffer and boiled for 5 min. A 20-μl volume of each sample was then separated on SDS-polyacrylamide gels, transferred to PVDF membranes (GE healthcare) and probed with anti-Myc antibody (Sigma-Aldrich).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Molecular identification of four *PhSLFs*.

Figure S2. PhSSK1 accumulates in the mature pollen.

Figure S3. Amino acid sequences and the predicted secondary structures of SSK1 proteins.

Figure S4. An unrooted neighbor-joining phylogeny tree of PhSSK1, AhSSK1 and Skp1-like proteins.

Figure S5. Schematic representation of the *PhSSK1* RNAi vector (*pBI101-LAT52-PhSSK1-RNAi*).

Figure S6. Expression analyses of PhSSK1 in pollen of T_0 transgenic lines.

Figure S7. Molecular identification of the style *S* defective line S_OS_O.

Figure S8. Predicted glycosylation sites in Ph*S*-RNase-S_{3L}/S_V/S_1/S_O.

Figure S9. Genotype identification of T_2 progeny from the outcrosses of the S_OS_O wild type compatible line with the T_1 transgenic lines as pollen donor.

Figure S10. Genotype identification of T_2 progeny derived from the outcrosses of the $S_{3L}S_{3L}$ wild type plants with the T_1 transgenic lines as pollen donor.

Table S1. A summary of yeast two-hybrid assays between the Skp1-like proteins and the *S*-locus F-box proteins of *A. hispanicum* and *P. hybrida*.

Table S2. Pollination analyses of seven independent T_0 transgenic lines.

Table S3. Primers used in this study.

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Sequence data from this article can be found in GenBank/EMBL databases under the following accession numbers: *PhSSK1* cDNA (FJ490176), *PhSSK1* genomic DNA (FJ490177), *PhCullin1* (FJ490178), *PhSKP1–1* (FJ490181), *PhSKP1–2* (FJ490179).

