The Plant Journal (2006) **46**, 780–793 doi: 10.1111/j.1365-313X.2006.02735.x

AhSSK1, a novel SKP1-like protein that interacts with the S-locus F-box protein SLF

Jian Huang[1,†], Lan Zhao[1,2,†], Qiuying Yang[1,2] 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 100080, China, and*
[2]*Graduate University of Chinese Academy of Sciences, Beijing 100049, China*

Received 31 October 2005; revised 12 January 2006; accepted 16 February 2006.
[*]For correspondence (fax +86 (10) 62537814; e-mail ybxue@genetics.ac.cn).
[†]These authors contributed equally to this work.

Summary

The *S*-locus F-box (SLF/SFB) protein, recently identified as the pollen determinant of S-RNase-based self-incompatibility (SI) in Solanaceae, Scrophulariaceae and Rosaceae, has been proposed to serve as the subunit of an SCF (SKP1–CUL1–F-box) ubiquitin ligase and to target its pistil counterpart S-RNase during the SI response. However, the underlying mechanism is still in dispute, and the putative SLF-binding SKP1-equivalent protein remains unknown. Here, we report the identification of AhSSK1, *Antirrhinum hispanicum* SLF-interacting SKP1-like1, using a yeast two-hybrid screen against a pollen cDNA library. GST pull-down assays confirmed the SSK1–SLF interaction, and showed that AhSSK1 could connect AhSLF to a CUL1-like protein. AhSSK1, despite having a similar secondary structure to other SKP1-like proteins, appeared quite distinctive in sequence and unique in a phylogenetic analysis, in which no *SSK1* ortholog could be predicted in the sequenced genomes of Arabidopsis and rice. Thus, our results suggest that the pollen-specific SSK1 could be recruited exclusively as the adaptor of putative SCF^{SLF} in those plants with S-RNase-based SI, providing an important clue to dissecting the function of the pollen determinant.

Keywords: self-incompatibility, SKP1, F-box, SLF, SSK1, *Antirrhinum*.

Introduction

Angiosperms, the most abundant and diverse plants on earth today, are characterized by the possession of true flowers and hence are commonly known as flowering plants. These plants are sessile and their male and female organs are close together within a single flower. Thus, these hermaphrodites have a strong tendency to self-pollinate, reducing the genetic diversity and fitness of their offspring. To avoid inbreeding and promote out-crossing, many plants have adopted self-incompatibility (SI) systems, through which incompatible (self or genetically related) pollen is recognized and rejected, whereas compatible (non-self) pollen is allowed to grow in the style and deliver the germ cells into the ovary for the double fertilization.

It was estimated by Brewbaker (1959), who conducted a broad survey of the angiosperms, that between one-third and half of flowering plants employ the SI systems to prevent self-fertilization. Among the various SI systems, gametophytic SI in the Solanaceae, Scrophulariaceae and Rosaceae appears to be the most common type, in which the specificity of the SI response is controlled by a single polymorphic locus, termed the *S*-locus. This locus carries two genes determining the specificity: one expressed in the pistil (pistil *S*) and another in the pollen (pollen *S*). When the haplotype of the pollen (haploid) *S* gene is the same as that of either *S* gene in the pistil (diploid), the haploid pollen tube will be unable to grow in the style and thus be rejected (reviewed by Kao and Tsukamoto, 2004; McClure, 2004; de Nettancourt, 2001).

The term S-RNase-based SI was coined after identification of S-RNase, the female determinant that was confirmed to play an *S*-haplotype-specific role in the SI response by transgenic experiments in both *Petunia* and *Nicotiana* (Lee *et al.*, 1994; Murfett *et al.*, 1994). S-RNases are expressed in the plant female tissues (pistil) and accumulate to high levels in the extracellular matrix, where they encounter the growing pollen tubes. It is now widely accepted that

S-RNases may enter the pollen cytoplasm in an *S*-haplotype-independent manner (Luu *et al.*, 2000) and exert their ribonuclease activity to trigger a cytotoxic effect in the cognate pollen tube (McClure *et al.*, 1990), while S-RNases in compatible pollination are inhibited by some mechanism involving pollen *S* products to ensure successful fertilization.

It took more than one decade and a great deal of effort to identify the pollen *S*, *SLF* (*S-locus F-box*) gene, until Lai *et al.* (2002) sequenced a 63.7 kb region containing the S_2-RNase of *Antirrhinum hispanicum*, a member of the Scrophulariaceae, and identified an anther-expressed gene that encodes a putative F-box protein (FBP). This gene was named *AhSLF-S*$_2$, and was then demonstrated to be the pollen *S* candidate as it was the sole allele-specific gene among the multiple F-box-encoding genes linked to the *S*-locus (Zhou *et al.*, 2003). Recently, *AhSLF-S*$_2$ was introduced into a self-incompatible line of *Petunia hybrida* (Qiao *et al.*, 2004b) and the transgenic plants became self-compatible, suggesting that SLF can control pollen function during the SI response. Meanwhile, Sijacic *et al.* (2004) reported specific SI breakdown induced by the introduction of an extra copy of *SLF* gene into the haploid pollen, after their thorough search of *PiSLF* genes and subsequent well-designed transformation experiments in *Petunia inflata*. Thus, *SLF* in *Antirrhinum* or *Petunia* was suggested to be the pollen *S* gene.

In the Rosaceae, a series of F-box-encoding genes were also found to be linked to the S-RNase genes, referred to as *SLF* in *Prunus mume* by Entani *et al.* (2003) and as *SFB* in *Prunus dulcis* by Ushijima *et al.* (2003), and were strongly proposed to be candidates for pollen determinants in SI. Intriguingly, the Rosaceae *SLF*/*SFB* genes exhibit a high level of allelic sequence diversity comparable with the S-RNases, unlike the pollen S proteins in the Solanaceae and Scrophulariaceae, which are less polymorphic, especially the latter. Furthermore, self-compatibility of *Prunus* pollen-part mutants examined by two groups (Sonneveld *et al.*, 2005; Ushijima *et al.*, 2004) was caused by the loss or significant alteration of *SFB* genes. In contrast, most (if not all) mutants found in the Solanaceae (Golz *et al.*, 2001; de Nettancourt, 2001; Tsukamoto *et al.*, 2005) and Scrophulariaceae (Y. Xue, unpublished data) lost SI simply because of duplication of pollen *S* genes, namely the *SLF* genes.

SLF/SFB protein contains an F-box domain at its N-terminus, which is characteristic for F-box proteins, the substrate receptor of SCF (SKP1-Cullin1-F-box) ubiquitin ligase, and is relevant to its possible role in recognizing the female S protein in the context of SI. The SCF ligase is composed of four subunits: SKP1, CUL1, F-box protein and a ring finger protein RBX1. This complex is widely spread throughout eukaryotes and absolutely necessary in a wide range of cellular processes (reviewed by Cardozo and Pagano, 2004; Petroski and Deshaies, 2005). As one type of ubiquitin ligase (E3), the SCF complex facilitates the transfer of ubiquitin from the ubiquitin-conjugating enzyme (E2) to a lysine residue of the substrate. After the consecutive addition of ubiquitin moieties, the substrate is tagged with a polyubiquitin chain, in other words it is undergoing a process called polyubiquitylation, and then is delivered to the 26S proteasome for unfolding and degradation (reviewed by Hershko and Ciechanover, 1998).

In plants, the SCF complex may have the potentially largest number of members among the various E3 families, as more than 700 F-box proteins and over 20 SKP1-like proteins have been annotated in Arabidopsis or rice, the two model plants with sequenced genomes. Also the SCF family is the most comprehensively studied so far. However, the current study of the plant SCF complex mostly focuses on the F-box proteins with conventional protein–protein interaction motifs, such as leucine-rich repeats and Kelch domains, at their C-termini (reviewed by Moon *et al.*, 2004; Smalle and Vierstra, 2004). In addition, these FBPs in Arabidopsis have been shown to interact with ASK1 or ASK2, the most strongly and widely expressed members of the Arabidopsis SKP1-like (ASK) proteins, via the F-box domain at the N-terminus (reviewed by Moon *et al.*, 2004; Smalle and Vierstra, 2004; see Table S1). In contrast, many FBPs, e.g. SLF/SFB or SON1 (Kim and Delaney, 2002), have no conventional domains at the C-termini and cannot yet be assigned an SKP1-like partner, consistent with the fact that their functions are largely unexplored (Gagne *et al.*, 2002; Wang *et al.*, 2004). Given that Rcy1 (Galan *et al.*, 2001) and Ctf13 (Russell *et al.*, 1999), which have irregular motifs compared with the other six FBPs in budding yeast, do not assemble into SCF complexes, it may be questioned whether the hundreds of FBPs in plants all serve to recognize the substrate protein in the ubiquitin–proteasome pathway.

The chromosomal linkage of the *SLF/SFB* gene to the *S-RNase* gene seemingly designates this F-box protein as a major player in the SI response and also suggests its interaction with S-RNases. This was demonstrated by Qiao *et al.* (2004a), who conducted experiments involving yeast two-hybrid screen and protein chemistry to identify the physical interaction between AhSLF-S_2 and S-RNases. They also suggested that the SLF could be assembled into an SCF complex to function in the ubiquitin–proteasome pathway. However, some SKP1-like proteins identified in *Antirrhinum*, i.e. FAPs (FIM-associated proteins; Ingram *et al.*, 1997), could not be determined to be the interacting partner of AhSLF during a yeast two-hybrid experiment (our unpublished data). Thus, the existence of an SLF-interacting SKP1-like protein and consequent SCF complex remains obscure.

Here we report the identification of AhSSK1, *Antirrhinum hispanicum* SLF-interacting SKP1-like1, through a yeast two-hybrid screen against a pollen cDNA library. GST pull-down experiments demonstrated that AhSSK1 could be the adaptor protein of a putative SCF^{SLF}. *AhSSK1* is specifically expressed in pollen and alternatively spliced. Moreover,

AhSSK1, an SKP1-like protein with an unusual primary structure, was proposed not to exist in many plants without S-RNase-based SI in a phylogenetic analysis. Although this novel *SKP1-like* gene is not linked to the *S*-locus as other important SI players such as *S-RNase* and *SLF* are, the SSK1 is probably only involved in SI signaling, as suggested by pollen bombardment experiments in which several dominant-negative *SKP1-like* derivatives were used. Further, the SSK1 identification may agree with the available genetic evidence in the Solanaceae and Scrophulariaceae, but suggests that the SI response in the Rosaceae could be different.

Results

AhSSK1 interacts with AhSLF-S_2 or AhSLF-S_5 in yeast

A yeast two-hybrid screen was used to identify proteins that may interact with AhSLF-S_2. The complete coding sequence of *AhSLF-S_2* was fused to the Gal4 DNA-binding domain (BD) and then introduced into *Saccharomyces cerevisiae* strain Y187 to generate the bait strain, while an *Antirrhinum* pollen cDNA library was constructed in *S. cerevisiae* strain AH109, resulting in the prey strain. We performed the screening by mating the two strains and identified 20 clones from 2 million diploid yeast cells for their enhanced growth on SD/-Ade-His-Leu-Trp medium and β-galactosidase activities.

Sequence analysis revealed that five clones corresponded to a novel SKP1-like protein, AhSSK1 (AhSLF-interacting SKP1-like1, gene accession number: DQ355479). To test its interacting specificity with various SLFs identified in our previous work (Zhou *et al.*, 2003), we transformed both the *AhSSK1* prey vector and individual *SLF* bait vector into AH109 for a growth test on SD minimal medium or into Y187 for β-galactosidase activity analysis (Figure 1a,b).

As expected, AhSSK1 specifically interacted with the full-length AhSLF-S_2 or its truncated version with the F-box domain (AhSLF-S_2-N) rather than that without the F-box (AhSLF-S_2-C). Moreover, it did not interact with the AhSLF paralogs, such as *AhSLF-S_1E*, *AhSLF-S_2C* and *AhSLF-S_4D* (Zhou *et al.*, 2003). Interestingly, it appeared that AhSSK1 selectively bound SLF protein of a certain *S*-haplotype, namely AhSLF-S_2 or S_5 rather than AhSLF-S_1 or S_4 (see Figure 1a,b). As FBP binds SKP1 through the F-box domain and all SLF proteins identified in plants with S-RNase-based SI, such as *Antirrhinum* and *Petunia*, display sequence polymorphism in this domain (Sijacic *et al.*, 2004; Zhou *et al.*, 2003), it can be deduced that AhSSK1 may confer a specific affinity to different SLF proteins and thus exert distinct functions in various SI backgrounds, although this would add an extra complexity to the SI system. However, it is more likely that this specificity occurs only in the yeast two-hybrid system and should be ruled out in pollen, as it was the *Antirrhinum* genes that were tested in this '*in vitro*' system.

The identified pollen *S* proteins of *Antirrhinum* could be categorized into two groups, AhSLF-S_1/S_4 and AhSLF-S_2/S_5, based on the above yeast two-hybrid results. This was

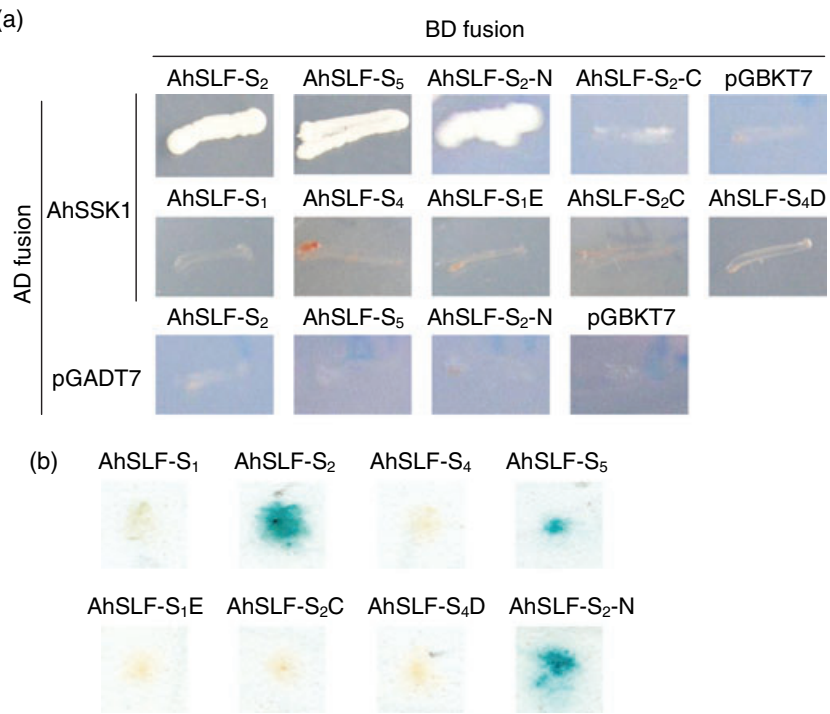

Figure 1. AhSSK1 interacts with AhSLF-S_2/S_5, but not with AhSLF paralogs.
(a) AhSSK1 interacted with AhSLF-S_2 or AhSLF-S_5 through binding the F-box domain at the N-terminus in yeast two-hybrid assays. The indicated combinations of bait (BD fusion) and prey (AD fusion) constructs were introduced into the yeast reporter strain AH109. Transformants were streaked on selective plates lacking adenine, histidine, leucine and tryptophan (SD/-Ade-His-Leu-Trp) and were examined for growth. The plates were photographed after 3 days incubation at 30°C.
(b) β-galactosidase activity of the yeast strain Y187 transformed with combinations of *AhSSK1* and *AhSLF* (orthologs and paralogs) constructs. The *AhSLF* vectors are indicated.

Table 1 A summary of yeast two-hybrid tests between *Antirrhinum* SKP1-like proteins and *S*-locus F-box proteins

	AhSLF-S_1	AhSLF-S_2	AhSLF-S_4	AhSLF-S_5	AhSLF-S_2-N	AhSLF-S_2-C	AhSLF-S_1E	AhSLF-S_2C	AhSLF-S_4D	pGBKT7
AhSSK1	–	+	–	+	+	–	–	–	–	–
FAP1	–	–	–	–	–	–	–	–	–	–
FAP2	–	–	–	–	–	–	–	–	–	–
pGADT7	–	–	–	–	–	–	–	–	–	–

Three SKP1-like proteins (AhSSK1, FAP1 and FAP2), seven S-locus F-box proteins (four ortholog alleles and three paralogs) and two truncated AhSLF-S_2 derivatives were included. The yeast growth on selective plates (SD/-Ade-His-Leu-Trp) after 3 days incubation at 30°C is indicated by + (strong growth) or – (no growth).

supported by the finding that two variable residues, I12T and C29L, exist in the 40amino acid F-box domains (see Figure S1). Point mutation assays and subsequent two-hybrid experiments indicated that only the latter (C29L) contributed to the allelic specificity of the SSK1–SLF interaction observed in yeast (data not shown).

We also examined whether FAPs, the SKP1-like proteins identified in *Antirrhinum majus*, could bind AhSLF in yeast. FAPs are a class of widely expressed SKP1-like proteins, which share high sequence identity to ASK1, a house-keeping SKP1 homolog in Arabidopsis (Zhao *et al.*, 2003). Also FAPs are suggested to be the ASK1 orthologs for its association with the FIMBRIATA, the *Antirrhinum* counterpart of ASK1-interacting UNUSUAL FLORAL ORGANS (Ingram *et al.*, 1997; Samach *et al.*, 1999). The two-hybrid results showed that no FAP protein was able to interact with any intact or truncated SLF protein in yeast (Table 1), suggesting a specific role for AhSSK1 in the SI response.

AhSSK1 represents a novel class of SKP1-like proteins

The canonical SKP1 proteins have 150–200 residues and can be divided into two major domains: the N-terminal section that interacts with CUL1 and the C-terminal fragment that binds the F-box domain (Zheng *et al.*, 2002), although acting as the adaptor in the SCF complex may not be the only function of SKP1-like proteins (Petroski and Deshaies, 2005). The 3D structural data of human SCF^{SKP2} revealed that human SKP1 and F-box protein SKP2 assemble into a four-layer sandwich: the H5, H6 and H7 helices of SKP1 on one side and a fraction of the SKP2 C-terminus on the other side, with the F-box and the H8 helix of SKP1 secured by the two sides (Schulman *et al.*, 2000). This SKP1–FBP interface is believed to be quite conserved from yeast to human, especially in its peripheral layers, while it also comprises some variable elements, predominantly in the sandwich center.

Plant SKP1 proteins, which share extensive similarity with their yeast and human counterparts in structure and function, are plentiful in the genome (21 in Arabidopsis and >25 in rice compared with only a single SKP1 in yeast and human), reminiscent of the staggeringly large numbers of plant F-box proteins (Gagne *et al.*, 2002; Risseeuw *et al.*, 2003). However, most SKP1-like proteins in Arabidopsis are highly conserved, even within the H8 domain. This suggests limited variety in the SKP1–FBP interaction. This limited polymorphism was also demonstrated by several reported yeast two-hybrid experiments (Risseeuw *et al.*, 2003).

AhSSK1, which shares 36.2% amino acid identity with HsSkp1 and 40.0% with ASK1, is also predicted to consist of eight helices (Figure 2). Thus, AhSSK1 could be an

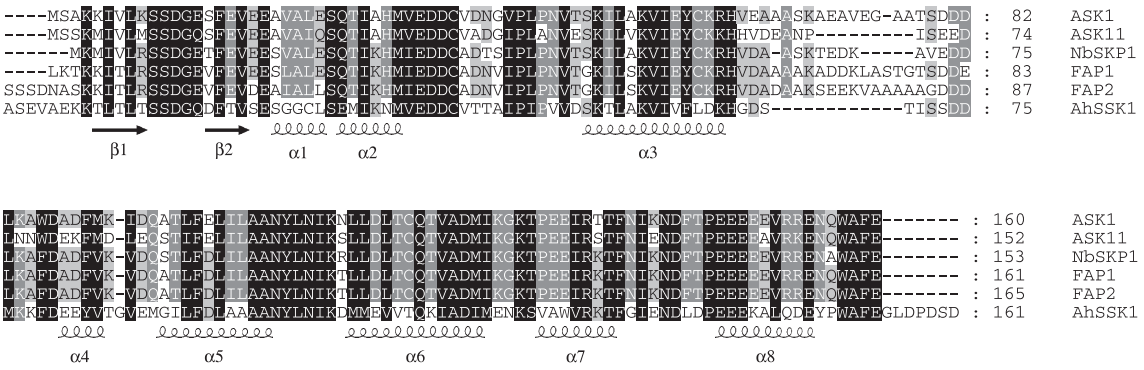

Figure 2. Sequence alignment and structural elements of AhSSK1 and several typical plant SKP1-like proteins.
ASKs, FAPs (FAP1, CAA75117; FAP2, CAA75118) and NbSKP1 (AAO85510) are from Arabidopsis, *Antirrhinum* and *Nicotiana*, respectively. Residues of 80–100% similarity are shaded in black; those of 60–80% similarity are denoted in dark gray; 30–60% similarity is indicated as black fonts on light-gray background. Numbers show the positions of amino acid residues. The arrow represents the β-strand and the squiggle symbolizes α-helix. The secondary structure prediction was based on AhSSK1, in parallel with other SKP1-like proteins.

Antirrhinum SKP1-like protein that binds AhSLF to form a sandwich structure in a similar way to other plant SKP1s. However, it is quite distinctive in its primary structure. Here we compared AhSSK1 with two typical ASKs (ASK1 and ASK11) and three SKP1-like proteins identified in plants with S-RNase-based SI: FAP1 and FAP2 (Ingram *et al.*, 1997) and NbSKP1 (Liu *et al.*, 2002). Significant differences between AhSSK1 and other SKP1-like proteins were revealed, especially in the C-termini (see Figure 2). Most prominently, AhSSK1 has a 7-residue tail following the H8 helix that is often the end of many plant SKP1-related proteins. Within the backbone of AhSSK1, numerous residues are also different from the conventional ones (Schulman *et al.*, 2000; Zheng *et al.*, 2002), such as Thr85, Met89, Ile91, Ala96, Glu109, Val111, Thr112, Lys114, Glu120, Asn121, Val124, Trp126, Gly132, Leu137, Asp138, Lys143, Gln146, Tyr149 and Pro150. It should be recognized that this rare distribution of amino acids not only covers H8, the capricious helix, but also H5, H6 and H7, the fairly conserved fragment that interacts directly with the F-box domain (Schulman *et al.*, 2000; Zheng *et al.*, 2002). Thus, it can be suggested that AhSSK1 is a novel SKP1-like protein, whose novel sequence might give rise to its specific affinity with SLF rather than other F-box proteins, such as UFO/FIM (Ingram *et al.*, 1997; Samach *et al.*, 1999), TIR1 (Gray *et al.*, 1999) and SLY1 (McGinnis *et al.*, 2003).

AhSSK1 may exist mainly in plants with S-RNase-based self-incompatibility

In addition to its distinguishing features in protein structure, AhSSK1 appears to be unique as a plant SKP1-like protein in the phylogeny relationship. We conducted a phylogenetic analysis of 59 *SKP1*-like genes based on amino acid sequences of 59 *SKP1*-like genes. The genes come from yeast (*ScSKP1*; Bai *et al.*, 1996), human (*HsSKP1*; Schulman *et al.*, 2000), *Brassica* (*BnSKP1*γ*1*, which is quite unusual; Drouaud *et al.*, 2000), Arabidopsis (*ASK1–ASK21*), rice (all related genes identified from the TIGR database), tobacco (all identified in this plant, that is *NbSKP1* and *NcSKP1*; Liu *et al.*, 2002) and *Antirrhinum* (*FAP*s and *AhSSK1*). As has been seen in other studies (Risseeuw *et al.*, 2003; Zhao *et al.*, 2003), some *SKP1*-like genes with detailed analyses, such as *ASK1*, *NbSKP1* and *FAP*s, were clustered, showing that they are orthologous or very closely homologous (Figure 3). Near this well-known clade were the remaining *ASK* genes (except *ASK20* and *ASK21*). Two non-plant *SKP1* genes, *ScSKP1* and *HsSKP1*, also lie near the *ASK* genes, suggesting that the majority of *SKP1*-like genes have experienced a similar evolutional process. Interestingly, *AhSSK1* was exceptional as a dicot gene for its distinctive location in this phylogeny tree. It was very far away from all *SKP1*-like genes with known function. In addition, we could not find an *AhSSK1* ortholog from any *SKP1*-related genes of Arabidopsis and rice in this analysis. This suggests that the *SSK1* gene might mainly exist in plants with S-RNase-based SI, such as those from Solanaceae, Scrophulariaceae and Rosaceae; in other words, *AhSSK1* appears to have no ortholog in many plants without S-RNase-based SI, such as Arabidopsis and rice.

AhSSK1 *is specifically expressed in pollen and has multiple transcripts as the result of alternative splicing and polyadenylation*

From the *AhSSK1* sequence obtained from the yeast two-hybrid screening, we identified an mRNA with a 486 bp protein coding sequence and a 447 bp 3′ untranslated region (Figure 4a). To confirm its identity, PCR analyses were carried out using primer pairs covering this 933 bp fragment. The PCR templates were *Antirrhinum* genomic DNA of two genotypes (S_1S_4 and S_2S_5) and complementary DNA from leaf, petal, style and pollen. The >2 kb PCR product was amplified from both genomic DNA samples (Figure 4b), and the sequencing result suggested that *AhSSK1* has >1.5 kb introns at its 3′ UTR. In addition, the sequences of genomic *AhSSK1*s from S_1S_4 and S_2S_5 plants were identical, although AhSSK1 interacted differently with the SLFs from S_1S_4 or S_2S_5 in budding yeast. Among the various cDNA templates, only that from pollen could produce an exact band for the 933 bp product (Figure 4b). Thus, this *AhSSK1* transcript was exclusively expressed in the *Antirrhinum* pollen.

To determine whether *AhSSK1* could have its >2 kb genomic DNA expressed in different transcripts, we performed a 3′ RACE experiment using the *Antirrhinum* pollen cDNA. Interestingly, the RACE results showed that there were three types of *AhSSK1* mRNA with different 3′ UTRs. They could be approximately classified into two classes, of which class A (accession number: DQ355479) represented exactly the mRNA mentioned above and class B resulted from transcription without splicing, although it had two members (B1 and B2, accession numbers: DQ355480 and DQ355481) as a consequence of alternative polyadenylation (see Figure 4a). Overall, the class B mRNAs were smaller in size, as the 3′ UTR of B1 was 226 bp and that of B2 was 302 bp. However, both of the two classes were pollen-specific (Figure 4b) and shared the same protein coding sequence, although under possibly differential regulation through their individual 3′ UTRs.

We next asked whether it was the different developmental stages that brought the various patterns to the 3′ UTR of *AhSSK1* mRNA; in other words, whether the mixture of multiple *AhSSK1* transcripts in 3′ RACE templates was caused by the indiscriminate collection and RNA preparation of young, mature and dehiscent anthers. Thus, we individually picked the unicellular microspores from the flower buds of 6 mm in size, the early bicellular pollen from those of 8 mm, the mid-bicellular pollen from those of 11 mm, and

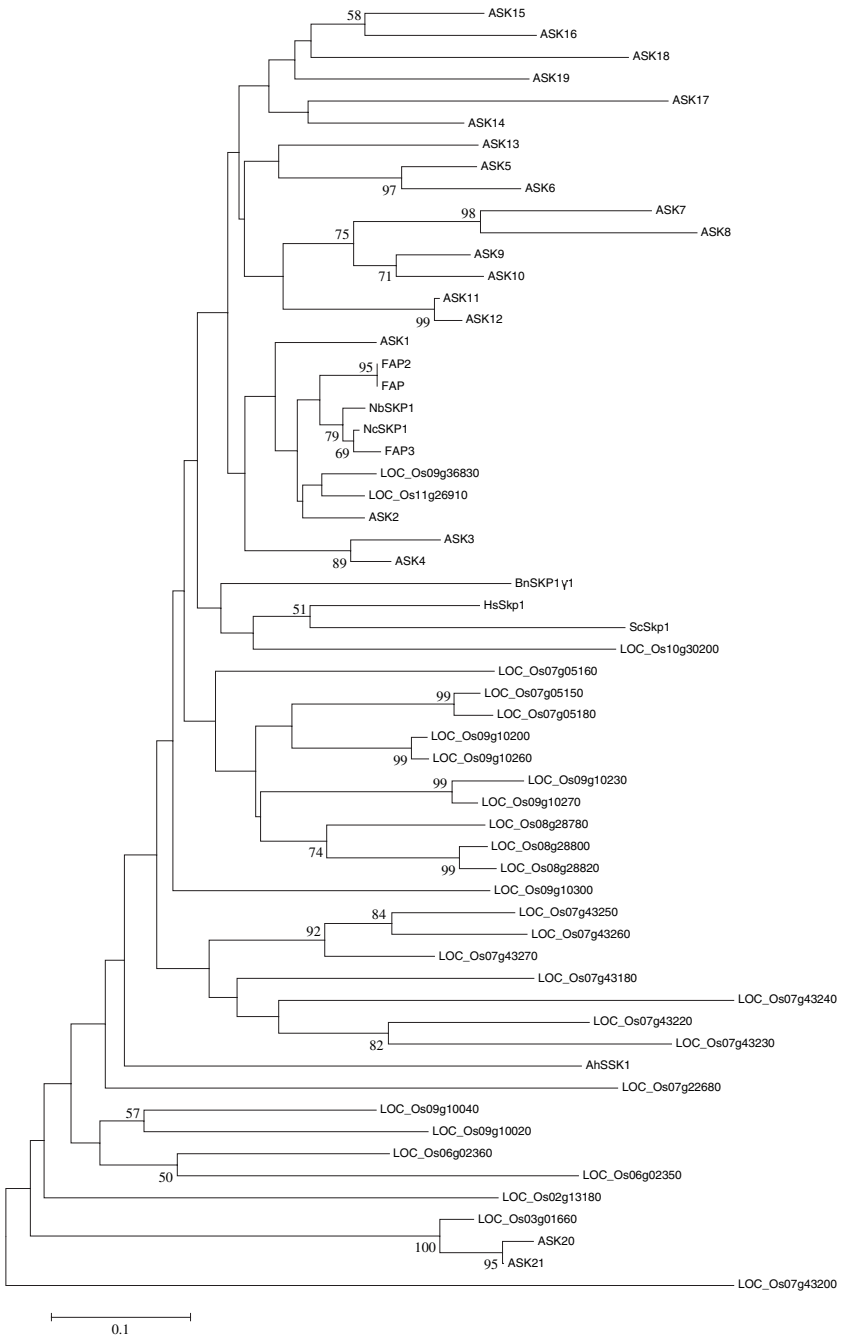

Figure 3. An unrooted neighbor-joining phylogeny tree of AhSSK1 and 58 other SKP1-like proteins.
The 58 deduced amino acid sequences are from budding yeast (ScSkp1, AAC49492), human (HsSkp1, AAH20798), Arabidopsis (ASK1–ASK21), rice (29 SKP1 homologues annotated or predicted by TIGR) and other higher plants (FAP1, CAA75117; FAP2, CAA75118; FAP3, CAA75119; NbSKP1, AAO85510; NcSKP1, AAC63273; BnSKP1γ1, AAF82795). Bootstrap values of 50% and above (based on 1000 neighbor-joining replicates) are given at branch nodes.

the nearly mature pollen from those of 14 mm, extracted RNA and performed RT-PCR against each sample. However, we could not ascribe a certain transcript to one or several developmental stages other than identifying when *AhSSK1* emerged as mRNA in pollen (Figure 4c). Our results showed that the *AhSSK1* gene, no matter which type of 3′ UTR it had, was predominantly expressed and accumulated in the bicellular pollen after pollen mitosis I, as rather low levels of RT-PCR products were detected for the pollen cDNA of the 6 mm flower buds, which were probably not uniform for microspores and possibly contaminated with some mature tissue.

The genomic organization of AhSSK1

To test whether *AhSSK1* is a haplotype-specific gene, we carried out Southern blot analysis of S_1S_4 and S_2S_5 genomic DNA using the >2 kb 3′ UTR (class A) genomic DNA of *AhSSK1* as probe. Three restriction enzymes (*Hin*dIII, *Eco*RI and *Bam*HI) were used, and multiple fragments were

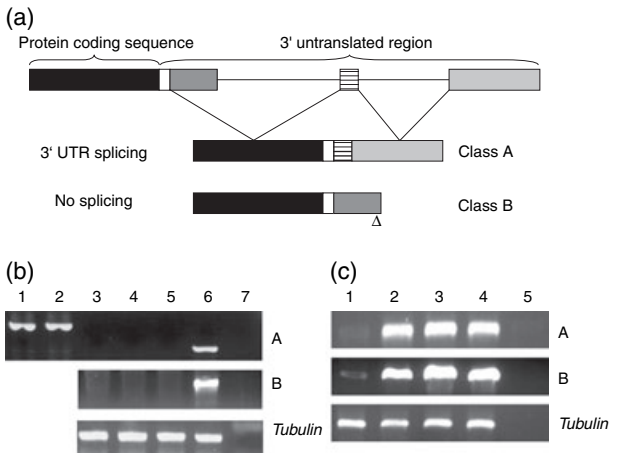

Figure 4. Alternative splicing and expression profile of *AhSSK1*.
(a) Comparison of multiple transcripts from the *AhSSK1* gene showing the events of alternative splicing and alternative polyadenylation within the 3′ untranslated region. The AhSSK1 coding sequence and 3′ UTR are indicated. Bars represent the exon candidates and thin lines indicate the intron regions. The spliced exons are connected by oblique lines and the polyadenylation sites are indicated by the Δ symbol. The resulting two types of *AhSSK1* mRNA (class A and B) are denoted.
(b) RNA was extracted from leaf and floral organs of *Antirrhinum*. Genomic DNA was prepared from S_1S_4 and S_2S_5 genotypes. The synthesized cDNA and genomic DNA were used as templates in PCR with *AhSSK1*-specific primers. The *tubulin* gene was included in RT-PCR to test whether the cDNA was equally loaded, integral, and not contaminated with genomic DNA. A and B indicate the specific primer pairs used for the two types of *AhSSK1* mRNAs, respectively. Lanes 1–7 represents S_1S_4 genomic DNA, S_2S_5 genomic DNA, leaf cDNA, petal cDNA, style cDNA, pollen cDNA and water, respectively.
(c) RNA was extracted from the *Antirrhinum* anthers of four developmental stages. A and B indicate the specific primer pairs used for the two types of *AhSSK1* mRNAs, respectively. Lanes 1–4 denote cDNA templates prepared from flower buds of 6, 8, 11 and 14 mm, respectively. Lane 5 is the negative control in which water was added.

detected in each lane (Figure 5a). The fragment polymorphism of *AhSSK1* hybridization was the same between S_1S_4 and S_2S_5, unlike that of the parallel experiment using S_2-*RNase* as the probe (Figure 5a). Thus, these results suggested that *AhSSK1* was perhaps multi-copy, lacking allelic specificity and not linked to the *S*-locus.

Next, we conducted fluorescence *in situ* hybridization (FISH) using a transformation-competent artificial chromosome (TAC) clone containing *AhSSK1* as probe to examine the chromosomal location of the *AhSSK1* gene. The TAC library was constructed with *A. hispanicum* of S_1S_5 genotype (Zhou *et al.*, 2003). PCR screening and PCR product sequencing revealed that several TAC clones including 91C10 contained the *AhSSK1* gene (data not shown). As shown in Figure 5(b), the clone 91C10 was located in the center region of the short arm of chromosome 1. As the *Antirrhinum S*-locus exists on chromosome 8 (Q. Yang, unpublished data), the *AhSSK1* gene is not linked to *SLF* or *S-RNase*. Although our FISH data could be not complete for *AhSSK1*, a possibly multi-copy gene, it still can be concluded that *AhSSK1* is not as polymorphic as the *SLF* and *S-RNase*, both of which are resident in the *S*-locus.

AhSSK1 is an adaptor that bridges AhSLF to CUL1-like protein

It was demonstrated that AhSSK1 could interact with AhSLF of S_2 (or S_5) rather than S_1 (or S_4) haplotype in the yeast two-hybrid assays (see Figure 1). However, this haplotype specificity could simply be caused by the limitations of the yeast two-hybrid system and may not occur in other systems. Thus, we conducted an *in vitro* binding assay to test whether AhSLF-S_1 or AhSLF-S_4 could bind AhSSK1 as the S_2 *or* S_5 alleles did in yeast. For convenience, we respectively fused the N-terminus (93 residues) of each AhSLF from the two representative genotypes, S_1 and S_2, to maltose binding protein (MBP) and fused AhSSK1 to glutathione S-transferase (GST), then expressed them in *Escherichia coli*. The *in vitro* binding experiments showed that AhSSK1 could be specifically associated with the N-terminus of each SLF (Figure 6a), no matter which *S*-haplotype the SLF was derived from, although the divergence of the F-box domains between S_1 (or S_4) and S_2 (or S_5) resulted in the totally different performance in the yeast two-hybrid assays.

We also carried out GST pull-down assays, using the purified GST–AhSSK1 and the crude pollen extracts of S_1S_4 or S_2S_5 genotype (Figure 6b). The extensively washed beads were eluted; then the eluate was immunoblotted and probed with anti-AhSLF antibody. AhSLF from both genotypes (S_1S_4 and S_2S_5) unselectively co-purified with GST–AhSSK1 (Figure 6c,d), confirming that the AhSSK1–AhSLF interaction could exist in *Antirrhinum* pollen of any S-haplotype.

To test whether AhSSK1 could be a component of the putative SCF^{SLF}, we investigated the interaction between AhSSK1 and *Antirrhinum* CUL1-like protein using a GST pull-down assay. Anti-AtCUL1 reactive bands were observed in the GST–AhSSK1 eluate and *Antirrhinum* pollen extracts (Figure 6e), migrating at approximately 85 kDa like AtCUL1 (Gray *et al.*, 1999). The results indicated that the bands correspond to an *Antirrhinum* CUL1-like protein, the proposed ortholog of Arabidopsis CUL1. Notably, the interaction between AhSSK1 and AhCUL1 involved neddylated CUL1 (RUB1-CUL1 or Nedd8-CUL1) much more preferentially than unmodified CUL1 (Figure 6e), consistent with the model that neddylation could release or prevent CUL1 from CAND1 and promote assembly of the SCF complex by facilitating the SKP1–CUL1 interaction (Goldenberg *et al.*, 2004). Taken together, our results suggest that AhSSK1 could interact with AhSLF and a CUL1-like protein to form an SCF-like complex in *Antirrhinum* pollen.

Next we investigated whether S-RNase could be present in the GST–AhSSK1 pull-down products from the mixed extracts of pollen and style, to test the accessibility of the putative SCF^{SLF} to S-RNase. As expected, S-RNase could be

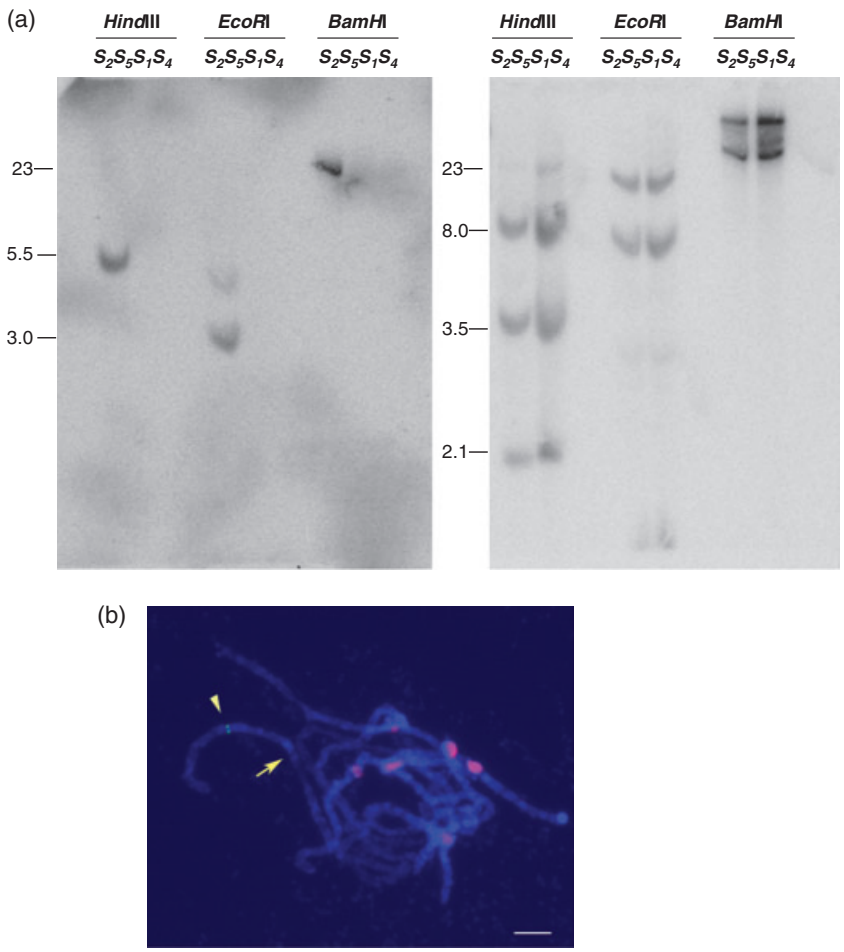

Figure 5. *AhSSK1* is not linked to the *S*-locus.
(a) Genomic DNA (8 μg) from the S_1S_4 or S_2S_5 lines of *Antirrhinum* was digested with *Hin*dIII, *Eco*RI and *Bam*HI, respectively. After separation by agarose gel electrophoresis and blotting, the DNA was hybridized with S_2-*RNase* cDNA (left) or *AhSSK1* 3′ UTR genomic DNA (right). The blot was washed at high stringency (0.1 × SSC and 0.5% SDS for 30 min twice). The numbers indicate the sizes of the hybridizing fragments in kilobase pairs.
(b) FISH analysis using a TAC clone containing *AhSSK1* as probe. Chromosomes (colored in blue) in a pachytene cell of *A. hispanicum* were probed by the *AhSSK1*-containing clone 91C10 (green signals, arrowhead) and anchored by CentA1 (red signals) to show the centromere positions. Chromosome identification was based on Zhang *et al.* (2005). The arrow indicates the centromere position of chromosome 1. Bar, 5 μm.

purified using GST–AhSSK1 (Figure 6f), perhaps through the recruitment of SSK1-interacting SLF. The results showed that S-RNase could be targeted by the supposed SSK1–SLF–Cul1–Rbx1 complex.

AhSSK1 is probably not required for pollen development except in the SI response

Given that AhSSK1 is exclusively expressed in pollen and associated with SLF proteins that may function only in the SI response, this SKP1-like protein could be exclusively used in the SI response, i.e. AhSSK1 could be not essential to pollen development when S-RNase was absent. To test this hypothesis, we bombarded *A. hispanicum* pollen with two *SKP1*-like derivatives and then germinated the bombarded pollen *in vitro*. in addition to AhSSK1, FAP1, which is the *Antirrhinum* ortholog of housekeeping ASK1, was also investigated in this experiment. To generate a dominant-negative effect, the CUL1-interacting domain of each SKP1-like protein (Zheng *et al.*, 2002) was deleted so the truncated AhSSK1 and FAP1, both of which could still recognize and bind their respective F-box proteins, might lose the normal functions and adversely interfere with the putative wild-type SCF ubiquitin ligases. Additionally we ensured that these mutated SKP1 proteins were predominantly expressed, by putting them under the control of the *LAT52* gene promoter (Twell *et al.*, 1991), and visually observed their expression through fusion of the fragments to β-glucuronidase (GUS) gene. The frequencies of transient expression were rather low, ranging from 0.01–0.03% for each bombarded construct. Pollen grains that expressed the truncated AhSSK1 (AhSSK1Δ) did not display any apparent phenotypic abnormalities, i.e. they were similar to the untransformed or pLAT52-GUS-introduced pollen in morphology and GUS staining pattern (Figure 7a,b). In contrast, only approximately 10% of the pollen grains that expressed the truncated FAP1 (FAP1Δ) could germinate, and the resulting pollen tubes did not reach a length comparable to that of the wild-type (Table 2 and Figure 7), while approximately 50% of the pollen grains expressing GUS or AhSSK1Δ-GUS germinated well. Moreover, in many pollen tubes that expressed FAP1Δ-GUS, the staining was quite slight (Figure 7c), compared with those producing AhSSK1Δ-GUS or GUS (Figure 7a,b).

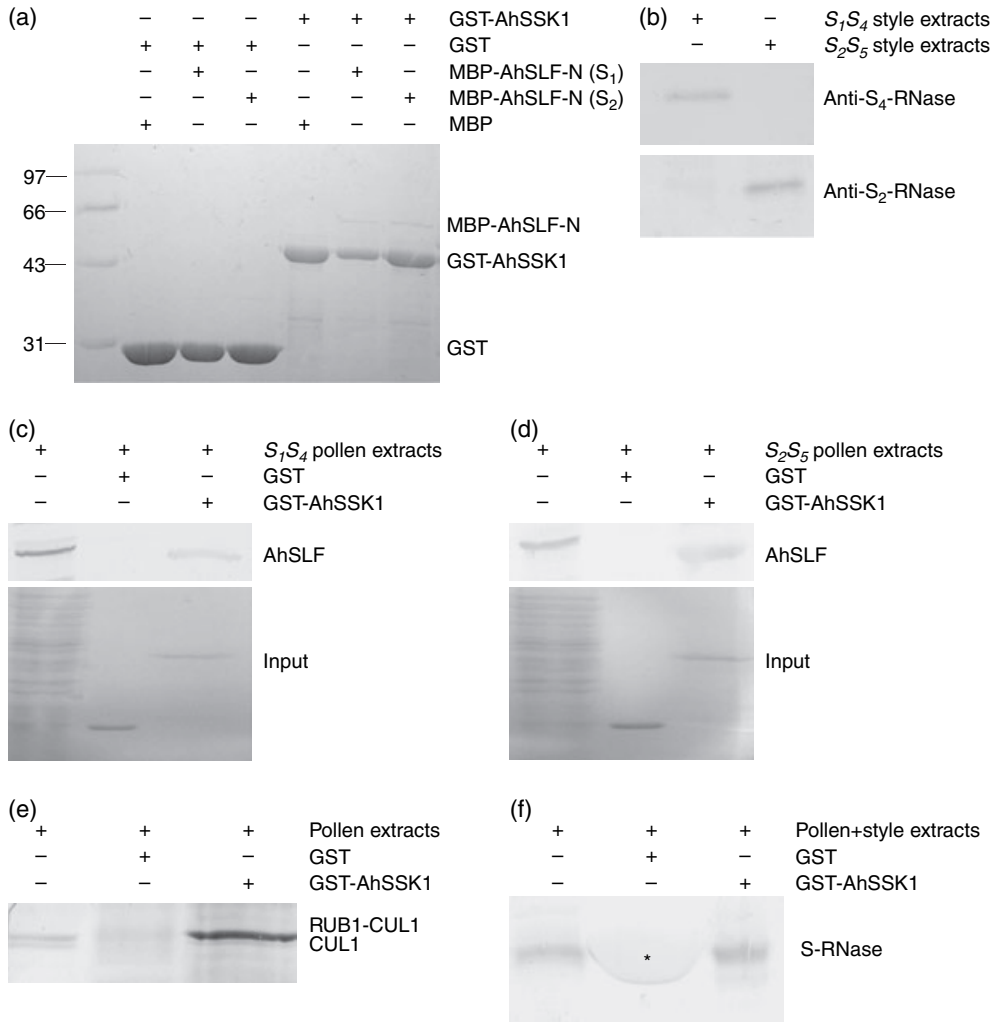

Figure 6. AhSSK1 connects AhSLF to CUL1-like protein.
(a) An *in vitro* pull-down assay showing that AhSSK1 interacts with AhSLF-S_1 or AhSLF-S_2. AhSSK1 was produced as a GST fusion protein in *E. coli* and bound to glutathione–Sepharose 4B resins, then incubated with lysate prepared from bacteria cultures of *MBP-AhSLF-N* (S_1 or S_2) recombinants. Following extensive washing of the beads, the proteins were eluted by 10 mM glutathione and resolved by SDS–PAGE. GST and MBP proteins were used as negative controls.
(b) Western analysis of style extracts using the allele-specific antibody against S_2- or S_4-RNase. Thus, the genotypes of certain lines were confirmed as indicated.
(c, d) The GST fusion proteins bound on glutathione–agarose beads were used in the pull-down assay with pollen extracts prepared from *Antirrhinum* lines of S_1S_4 or S_2S_5 genotypes as confirmed in (b). The lower panels denote the input proteins stained with Ponceau S. The bound proteins were immunoblotted and probed with anti-AhSLF antibody.
(e) The pollen extracts used were prepared from plants of various genotypes. This pull-down assay was probed with anti-AtCUL1 antibody.
(f) A combination of *Antirrhinum* pollen and style extracts in the ratio 1:1 was incubated with GST fusion proteins bound on glutathione–agarose beads. The anti-S-RNase serum was used in the Western experiment. The asterisk denotes GST protein that is similar to S-RNase in molecular weight and able to react with anti-*E. coli* antibodies in antiserum. This non-specific band indicates equal loading of GST fusion proteins.

This could be due to the possibility that, as FAP1 is a housekeeping gene like its ortholog ASK1, the dominant-negative effect caused by FAP1Δ could be deleterious to the pollen development and germination; thus, only the pollen grains that adopted mechanisms to reduce the FAP1Δ accumulation could diminish the adverse effect and germinate moderately, resulting in pollen tubes with weakened GUS staining (Figure 7c). In conclusion, our results suggest that *AhSSK1* is not essential for normal pollen development.

Discussion

AhSSK1 is a novel plant SKP1-like protein

The remarkably limited identity to other SKP1-like proteins in plants as well as its unique position in the phylogenetic tree of the plant SKP1 family indicate that AhSSK1 belongs to a novel class of SKP1-like proteins that has not been reported previously. The apparent distinction of AhSSK1 could be partly due to its interaction with AhSLF. This SLF/SFB

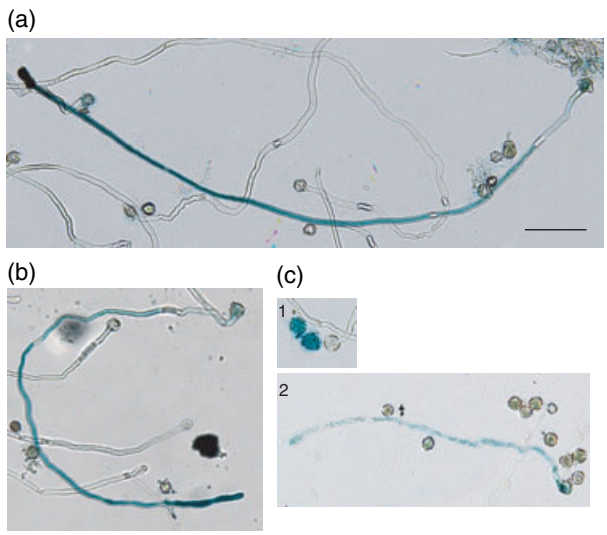

Figure 7. AhSSK1 is not essential for pollen germination *in vitro*.
The mature *Antirrhinum* pollen grains were bombarded with individual constructs, cultured at 20°C for 16 h and stained for GUS activity. The photographs are taken from representative samples transformed with (a) pLAT52-AhSSK1Δ-GUS, (b) pLAT52-GUS and (c) pLAT52-FAP1Δ-GUS. In (c), '1' indicates the germinating pollen tube with light staining and '2' denotes the majority of stained cells which could not germinate to produce visible pollen tubes. Bar, 100 μm in all cases.

Table 2 The germination frequency and pollen tube length for each bombarded construct

Bombarded constructs	Germination frequency of transgenic pollen (%)	Length of transgenic pollen tube (μm)
pLAT52-AhSSK1Δ-GUS	46.1 ± 3.6	875 ± 315
pLAT52-FAP1Δ-GUS	13.1 ± 2.0	535 ± 316
pLAT52-GUS	46.6 ± 4.3	836 ± 156

protein is quite exceptional when compared with other plant F-box proteins with known function (Dieterle *et al.*, 2001; Dill *et al.*, 2004; Fu *et al.*, 2004; Gray *et al.*, 2001; Guo and Ecker, 2003; Harmon and Kay, 2003; Ingram *et al.*, 1997; Kim and Delaney, 2002; Potuschak *et al.*, 2003; Samach *et al.*, 1999; Sasaki *et al.*, 2003; Woo *et al.*, 2001; Xu *et al.*, 2002; Yasuhara *et al.*, 2004). First, except for SLF/SFB, SON1, UFO/FIM and SLY1 (a very small FBP containing only 151 amino acids), most studied FBPs (TIR1, COI1, EBF1/EBF2, EID1, AFR, ADO/FKF/LKP/ZTL and ORE9/MAX2) have conventional protein–protein interaction motifs at the C-termini, such as leucine-rich repeats, Kelch repeats or leucine zippers (see Table S1). Second, SLF/SFB bears a very limited identity to other FBPs in the F-box domain, except SON1 (see Figure S2). It is noteworthy that SLF/SFB shares extensive identity to SON1 in both the C-terminus and F-box domain. Third, almost all FBPs mentioned above can use ASK1/ASK2 to form SCF ubiquitin ligases, except SLF/SFB and SON1, for which the SKP1 partners had not been reported (see Table S1). These unusual features of SLF/SFB give its partner SSK1 a remarkable distinction from other FBP-interacting SKP1 homologs, such as ASK1 and ASK2.

Thus, it could be suggested that AhSSK1, which is derived from the same origin as other plant SKP1-related proteins, is a novel SKP1-like protein in terms of its primary structure and phylogenetic position. It will be interesting to investigate whether AhSSK1 has an alternative function, different from ASK1 and FAPs.

SSK1, a new clue for self-incompatibility

The SI response can be initiated by the specific recognition/interaction of polymorphic S proteins between the pistil and pollen or pollen tube. This can elicit the orchestration of multiple genes, many of which are outside the *S* locus and monomorphic, resulting in self-pollen rejection and non-self-pollen acceptance. Our results suggested that the pollen-expressed AhSSK1 could be an indispensable player involved in SI response, for its adaptor role of associating AhSLF with CUL1, although *AhSSK1* is not linked to the *S*-locus.

Recently, the breakdown of self-incompatibility in the Solanaceae and Rosaceae has been highlighted (for details, please see the introduction and Hauck *et al.*, 2006) in terms of the apparent discrepancy in the breakdown causes between the families. In the Solanaceae, it is suggested that the pollen *S* product should inhibit all non-self S-RNases to promote cross-pollination, but leave the cognate S-RNase intact to hamper self-fertilization. Thus, the two different pollen *S* products in one pollen grain could inhibit all S-RNases. This model also predicts that the pollen *S* deletion will be lethal, because of the inability to inhibit any S-RNases. In contrast, as the Rosaceae model suggests, the deletion of the pollen *S* gene results in self-compatibility, because the pollen *S* product prevents inactivation of the self S-RNase by a general S-RNase inhibitor, through the *S*-haplotype-specific interaction between SFB and its cognate S-RNase (Sonneveld *et al.*, 2005). Combined with the differences in the S-RNase structures of the Solanaceae and Rosaceae (Matsuura *et al.*, 2001), these findings suggest divergence of the SI mechanisms between the Solanaceae and Rosaceae.

The identification of AhSSK1 provides more insights into the presence of the SCF^{SLF} complex in SI response. For the Solanaceae model (conceivably also suitable for the Scrophulariaceae), a putative SCF^{SLF} would be favorable, as the non-self S-RNase could be degraded after specific polyubiquitylation by the SCF ubiquitin ligase. However, as the Rosaceae model suggests, the SLF/SFB-interacting SKP1-like and the corresponding $SCF^{SLF/SFB}$ are not necessary, as the Rosaceae SLF/SFB plays a role of protection rather than destruction for the S-RNase. Thus it is quite important to

isolate the *AhSSK1* counterparts in the Solanaceae and Rosaceae, especially the latter. An SKP1-like protein that can interact with SLF/SFB in the Solanaceae or Rosaceae would confirm the universality of the SKP1–SLF/SFB interaction, in the light of the differences between the Solanaceae and Rosaceae.

However, a caveat should be issued for the supposition that SLF acts as a component of a canonical SCF ubiquitin ligase to target S-RNase for degradation. To date, most studies on SI mechanisms related to the ubiquitin–proteasome pathway have been carried out *in vitro* and lacked genetic evidence, partly due to the difficulty in mutant isolation and plant transformation. For *SSK1*, subsequent work should be extend from *A. hispanicum*, which is difficult to transform, to the Solanaceae, in which transgenic analysis and epitope tagging can be achieved through transformation for reverse genetics and biochemical analyses.

A new paradigm for the SKP1–F-box protein interaction

Along with nematodes, plants have more than 20 *SKP1*-like genes, while yeast and human each have a single *SKP1*. Compared with *Caenorhabditis elegans*, which also contains 21 *SKP1*-related genes in its genome (Nayak *et al.*, 2002; Yamanaka *et al.*, 2002), Arabidopsis has less divergent *ASK*s, of which the H8 helices are also not as variable as supposed (Risseeuw *et al.*, 2003). However, the rice SKP1-like proteins are not so homogenous in terms of amino acid sequences and phylogenetic relationship, indicating a difference between Arabidopsis and other plants. Moreover, the numerous FBPs identified in plant genomes, including those that contain unusual F-box domains and unknown C-terminus motifs such as SLF/SFB, have not yet been explored for their interaction with certain SKP1-like proteins. Here, the identification of *AhSSK1* in the dicot plant *Antirrhinum* provides a novel paradigm for the SKP1-FBP interaction.

Although it has a 7-residue tail and an unusual composition of amino acids in its backbone, AhSSK1 could be folded with an α/β structure that is similar to those of other identified SKP1s, such as HsSkp1, ASKs and FAPs. In addition, the GST pull-down results showed that AhSSK1 could interact with AhSLF and CUL1-like molecules. These data support the SSK1–SLF interaction and strongly suggest the possibility of an SCF complex that is composed of SSK1, CUL1, SLF and Rbx1. Some questions could be raised for the unusual AhSSK1. What could be the possible role of the AhSSK1 tail? Why should SLF recruit such an SKP1-like protein to function in SI? Could the SSK1–SLF interface play other roles in an SCF complex, such as an alternative mechanism regulating proteasomal degradation? What is the fate of S-RNase after its interaction with such an unusual complex? In summary, it is possible that SSK1 performs roles other than being an adaptor in the supposed SCF^{SLF}, which are related to but distinct from those of ASK1 and FAPs.

So far abundant FBPs and SKP1-like proteins have been found in the completed genomic sequences of Arabidopsis and rice, with a few genes investigated for their functions. With the progress of the SKP1–FBP studies in yeast and animals, this interface has been demonstrated to be neither restricted to functioning in SCF ubiquitin ligase nor limited to recruitment of target substrates for proteolysis. Furthermore, for plants that have more diverse, plentiful FBPs and SKP1-like proteins, the details of SKP1–F-box interface and SCF complex will be more complicated, already partly demonstrated by the recent discovery of TIR's new role as the auxin receptor (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). In fact, many plant FBP 'orphans' contain unidentified protein–protein interaction motifs at the C-terminus and the F-box domain to which a definite plant SKP1 homolog has not been allocated, leaving their functions largely elusive. It is not even clear whether the current SCF model is representative of the numerous F-box and SKP1-like proteins in plants. Studies of SSK1 function in SI will partially enable this question to be addressed, and also provide new clues to the molecular mechanism of S-RNase-based self-incompatibility.

Experimental procedures

Plant material

Antirrhinum self-incompatible lines derived by inter-specific crosses between *A. majus* and *A. hispanicum*, as well as their growth conditions, have been described previously (Xue *et al.*, 1996).

Plasmid constructs

Yeast two-hybrid bait vectors were constructed by ligating *Eco*RI- and *Pst*I-digested fragments of cDNA (*AhSLF-S_1*, *AhSLF-S_2*, *AhSLF-S_4*, *AhSLF-S_5*, *AhSLF-S_1E*, *AhSLF-S_2C*, *AhSLF-S_4D*, *AhSLF-S_2-N* and *AhSLF-S_2-C*) to pGBKT7 (Clontech, Palo Alto, CA, USA). *AhSLF-S_2-N* encodes the 93 amino acid peptide of the AhSLF-S_2 N-terminus (1–93) and *AhSLF-S_2-C* encodes the 121 amino acid peptide of the AhSLF-S_2 C-terminus (256–376). Prey vectors were constructed by ligating *Eco*RI- and *Xho*I-digested fragments of cDNA (*FAP1*, *FAP2* and *AhSSK1*) to pGADT7 (Clontech).

The *AhSLF-S_2-N* and *AhSLF-S_1-N* cDNA were respectively cloned in-frame into the MBP expression vector pMAL-c2x (NEB, Beverly, MA, USA). The *AhSSK1* cDNA was cloned in-frame into the GST expression vector pGEX-4T-1 (Amersham Biosciences, Uppsala, Sweden).

The pLAT52-GUS vector (Twell *et al.*, 1991) was used as a control in bombardment experiments. The vector was also restricted with *Xho*I and *Sma*I and ligated to the truncated *AhSSK1* (226–474) or *FAP1* (259–483) to generate pLAT52-AhSSK1Δ-GUS or pLAT52-FAP1Δ-GUS.

Antibodies

Anti-S-RNase and anti-AtCUL1 antibodies were as described previously (Qiao *et al.*, 2004a); anti-AhSLF-S_2 antibody was raised in rabbit using MBP-AhSLF-S_2-N as the antigen.

Yeast two-hybrid screening and assays

A yeast two-hybrid screening was carried out as described in the BD Matchmaker™ Library Construction & Screening Kits User Manual (Clontech). The bait strain was generated by introducing the pGBKT7-AhSLF-S_2 into *S. cerevisiae* strain Y187. An *Antirrhinum* pollen cDNA library was constructed in *S. cerevisiae* strain AH109, the prey strain, by recombination-mediated cloning in yeast. About 2×10^6 yeast transformants were screened on the selective medium SD/-Ade-His-Leu-Trp. Twenty clones were able to grow on this medium and displayed positive β-galactosidase activity. The prey plasmids were extracted, transformed into *E. coli*, and re-transformed with pGBKT7-AhSLF-S_2 into AH109 or Y187 to test the growth condition or β-galactosidase activity. The positive candidates were sequenced.

To investigate or confirm the interaction of individual prey and bait, we transformed the yeast AH109 strain with various combinations of bait and prey plasmids on SD/-Trp/-Leu medium. After 3 days, the transformants were streaked on SD/-Ade-His-Leu-Trp medium to test whether the combination could produce positive results. To test the β-galactosidase activities, the plasmids were transformed into the yeast Y187 strain on SD/-Trp/-Leu medium. The resulting Y187 clone was used for a β-galactosidase filter assay according to the manufacturer's protocol.

GST pull-down

GST and GST–AhSSK1 fusion protein were expressed in BL21. Induced bacteria were lysed by sonication in PBS. Clarified lysates were incubated with glutathione–Sepharose 4B beads (Amersham Biosciences) for 1 h at 25°C in PBS. Beads were then washed four times with 10 bed volumes of PBS.

The MBP fusion proteins were produced in BL21(DE3). The cleared lysates were incubated with 10 μg purified GST or GST–AhSSK1 protein bound to 20 μl stacked glutathione–Sepharose 4B beads for 1 h at 25°C. After the beads were washed four times with PBS, the pull-down proteins were eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) and resolved by SDS–PAGE.

Crude extracts of *Antirrhinum* pollen were prepared from open flowers of certain lines or multiple lines in lysis buffer containing 50 mM Tris-Cl (pH 8.0), 10% glycerol, 50 mM NaCl, 1 mM DTT, 1 mM PMSF, 10 μM MG132 and 1 × protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The cleared pollen crude extracts were incubated with 20 μg purified GST or GST–AhSSK1 protein bound to 30 μl stacked glutathione–Sepharose 4B beads for 3 h at 4°C. After the beads were washed four times with lysis buffer containing 0.1% Triton X-100, the pull-down proteins were eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The eluates were separated on 7.5% SDS–polyacrylamide gels, transferred to PVDF membranes (Amersham Biosciences) and probed with anti-AtCUL1 or anti-AhSLF-S_2 antibodies. The similar procedure was applied for S-RNase detection in the GST–AhSSK1 pull-down assay, except that the pollen and style extracts were combined in the ratio 1:1 to generate the crude protein extracts.

DNA gel blotting analyses

Genomic DNA isolation was performed as described previously (Xue *et al.*, 1996). The digested *Antirrhinum* genomic DNA (8 μg) was separated on a 0.8% agarose gel, and transferred to Hybond-N+ membrane (Amersham Biosciences). Prehybridization, hybridization and washing of the blot were performed according to the manufacturer's protocol. Probes were labeled with ^{32}P by random priming using the Prime-α-Gene labeling system (Promega, Madison, WI, USA).

TAC screening and fluorescence in situ *hybridization*

The TAC library (Zhou *et al.*, 2003), constructed from a self-incompatible line with S_1S_5 alleles, was screened to isolate the *AhSSK1*-containing clones using the primers 5′-GCAAGTGAAGTAGCCGAAAAG–3′ and 5′-TTATGAATTTAAGTTCTGCTAA-3′, as described by Zhou *et al.* (2003). The positive clones were labeled as FISH probes.

The chromosome preparation and FISH procedure were performed according to the method described by Zhang *et al.* (2005). Chromosomes and FISH signal images were captured with an Olympus BX61 fluorescence microscope in conjunction with a microCCD camera.

RT-PCR and genomic DNA PCR

Total RNA and genomic DNA were prepared as previously described (Lai *et al.*, 2002). The cDNA was produced using the SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and a poly-dT primer. The primers used to amplify *AhSSK1* cDNA of class A and >2 kb genomic DNA were 5′-GCAAGTGAAGTAGCCGAAAAG-3′ and 5′-GGACACAAAGTTTCTTAAGATAAT C-3′. The primers used to amplify *AhSSK1* cDNA of class B were 5′-GCAAGTGAAGTAGCCGAAAAG-3′ and 5′-TTATGAATTTAAGTTCTGCTAA-3′. PCR products were resolved by gel electrophoresis and sequenced to confirm their identity.

Microprojectile bombardment of pollen and GUS staining

The helium-driven PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA, USA) was used for the biolistic transformation of mature pollen grains of *Antirrhinum*. The pollen was collected from flower buds that had fully matured but had not undergone anthesis, and was resuspended at 1×10^6 cells in 100 μl of germination medium (0.1 mg ml^{-1} H_3BO_3, 0.71 mg ml^{-1} $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.2 mg ml^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.1 mg ml^{-1} KNO_3, 10% sucrose, 0.5 mg ml^{-1} MES, pH 5.9). The suspension was spread in the middle of a 5 cm Petri dish to create an even monolayer of cells. Each pollen monolayer was bombarded three times as described previously (Golovkin and Reddy, 2003), and 5 ml of germination medium was added. The plates were then left in the dark without shaking in a humid chamber at 20°C. After 16 h, the pollen tubes were collected by brief centrifugation at 100–200 ***g*** and subjected to GUS staining in a substrate solution containing 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.5 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) and 2 mM each of potassium ferricyanide and potassium ferrocyanide at 37°C for 12 h.

Alignment and phylogenetic analysis

Sequence alignment of the SKP1-like proteins was performed using ClustalW (Chenna *et al.*, 2003). Secondary structure prediction was carried out using the PredictProtein server (Rost *et al.*, 2004). Phylogenetic analysis of the *SKP1* gene family based on amino acid sequences was carried out using a neighbor-joining (NJ) method with MEGA version 3.0 (Kumar *et al.*, 2004). NJ analysis was done with the 'complete deletion' option selected. Support for each node was tested with bootstrap analysis, 1000 replicates for NJ, using random input order for each replicate.

Acknowledgements

We would like to thank Drs Enrico Coen and Rosemary Carpenter for providing *Antirrhinum* plants and constant support, Sheila McCormick for providing LAT52 promoter, and Zhukuan Cheng for assistance with the FISH experiment. This work was supported by the Chinese Academy of Sciences and the National Natural Science Foundation of China (30221002).

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Alignment of F-box domains of AhSLFs from four *S*-haplotypes in *Antirrhinum*.

Figure S2. Comparison of the F-box domains of some typical FBPs.

Table S1 A summary of the plant F-box proteins with identified function

This material is available as part of the online article from http://www.blackwell-synergy.com

References

Bai, C., Sen, P., Hofman, K., Ma, L., Goebel, M., Harper, W. and Elledge, S. (1996) *SKP1* connects cell cycle regulation to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*, **86**, 263–274.

Brewbaker, J.L. (1959) Biology of the angiosperm pollen grain. *Indian J. Genet. Plant Breed.* **19**, 121–133.

Cardozo, T. and Pagano, M. (2004) The SCF ubiquitin ligase: insights into a molecular machine. *Nat. Rev. Mol. Cell. Biol.* **5**, 739–753.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G. and Thompson, J.D. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**, 3497–3500.

Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005) The F-box protein TIR1 is an auxin receptor. *Nature*, **435**, 441–445.

Dieterle, M., Zhou, Y.C., Schafer, E., Funk, M. and Kretsch, T. (2001) EID1, an F-box protein involved in phytochrome A-specific light signaling. *Genes Dev.* **15**, 939–944.

Dill, A., Thomas, S.G., Hu, J., Steber, C.M. and Sun, T.P. (2004) The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *Plant Cell*, **16**, 1392–1405.

Drouaud, J., Marrocco, K., Ridel, C., Pelletier, G. and Guerche, P. (2000) A *Brassica napus skp1*-like gene promoter drives GUS expression in *Arabidopsis thaliana* male and female gametophytes. *Sex. Plant Reprod.* **13**, 29–35.

Entani, T., Iwano, M., Shiba, H., Che, F.S., Isogai, A. and Takayama, S.F.-S., Isogai, A. and Takayama, S. (2003) Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen-expressed F-box gene with allelic diversity. *Genes Cells*, **8**, 203–213.

Fu, X., Richards, D.E., Fleck, B., Xie, D., Burton, N. and Harberd, N.P. (2004) The Arabidopsis mutant sleepy1$^{gar2\text{-}1}$ protein promotes plant growth by increasing the affinity of the SCF^{SLY1} E3 ubiquitin ligase for DELLA protein substrates. *Plant Cell*, **16**, 1406–1418.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M. and Vierstra, R.D. (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **99**, 11519–11524.

Galan, J.M., Wiederkehr, A., Seol, J.H., Haguenauer-Tsapis, R., Deshaies, R.J., Riezman, H. and Peter, M. (2001) Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. *Mol. Cell. Biol.* **21**, 3105–3117.

Goldenberg, S.J., Cascio, T.C., Shumway, S.D., Garbutt, K.C., Liu, J., Xiong, Y. and Zheng, N. (2004) Structure of the Cand1–Cul1–Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. *Cell*, **119**, 517–528.

Golovkin, M. and Reddy, A.S.N. (2003) A calmodulin-binding protein from *Arabidopsis* has an essential role in pollen germination. *Proc. Natl Acad. Sci. USA*, **100**, 10558–10563.

Golz, J.F., Oh, H.-Y., Su, V., Kusaba, M. and Newbigin, E. (2001) Genetic analysis of *Nicotiana* pollen-part mutants is consistent with the presence of an *S*-ribonuclease inhibitor at the *S* locus. *Proc. Natl Acad. Sci. USA*, **98**, 15372–15376.

Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H. and Estelle, M. (1999) Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Dev.* **13**, 1678–1691.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001) Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. *Nature*, **414**, 271–276.

Guo, H. and Ecker, J.R. (2003) Plant responses to ethylene gas are mediated by SCF$^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. *Cell*, **115**, 667–677.

Harmon, F.G. and Kay, S.A. (2003) The F box protein AFR is a positive regulator of phytochrome A-mediated light signaling. *Curr. Biol.* **13**, 2091–2096.

Hauck, N.R., Yamane, H., Tao, R. and Iezzoni, A.F. (2006) Accumulation of non-functional S-haplotypes results in the breakdown of gametophytic self-incompatibility in tetraploid *Prunus*. *Genetics*, **172**, 1191–1198.

Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–479.

Ingram, G.C., Doyle, S., Carpenter, R., Schultz, E.A., Simon, R. and Coen, E.S. (1997) Dual role for *fimbriata* in regulating floral homeotic genes and cell division in *Antirrhinum*. *EMBO J.* **16**, 6521–6534.

Kao, T.-h. and Tsukamoto, T. (2004) The molecular and genetic bases of S-RNase-based self-incompatibility. *Plant Cell*, **16**, S72–S83.

Kepinski, S. and Leyser, O. (2005) The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature*, **435**, 446–451.

Kim, H.S. and Delaney, T.P. (2002) Arabidopsis SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. *Plant Cell*, **14**, 1469–1482.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* **5**, 150–163.

Lai, Z., Ma, W., Han, B., Liang, L., Zhang, Y., Hong, G. and Xue, Y. (2002) An F-box gene linked to the self-incompatibility (*S*) locus of *Antirrhinum* is expressed specifically in pollen and tapetum. *Plant Mol. Biol.* **50**, 29–42.

Lee, H.-S., Huang, S. and Kao, T.-h. (1994) *S* proteins control rejection of incompatible pollen in *Petunia inflata*. *Nature*, **367**, 560–563.

Liu, Y., Schiff, M., Serino, G., Deng, X.-W. and Dinesh-Kumar, S.P. (2002) Role of SCF ubiquitin-ligase and the COP9 signalosome in the *N* gene-mediated resistance response to *Tobacco mosaic virus*. *Plant Cell*, **14**, 1483–1496.

Luu, D.-T., Qin, X., Morse, D. and Cappadocia, M. (2000) *S*-RNase uptake by compatible pollen tubes in gametophytic self-incompatibility. *Nature*, **407**, 649–651.

Matsuura, T., Sakai, H., Unno, M., Ida, K., Sato, M., Sakiyama, F. and Norioka, S. (2001) Crystal structure at 1.5 Å resolution of *Pyrus pyrifolia* pistil ribonuclease responsible for gametophytic self-incompatibility. *J. Biol. Chem.* **276**, 45261–45269.

McClure, B. (2004) S-RNase and SLF determine S-haplotype-specific pollen recognition and rejection. *Plant Cell*, **16**, 2840–2847.

McClure, B.A., Gray, J.E., Anderson, M.A. and Clarke, A.E. (1990) Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. *Nature*, **347**, 757–760.

McGinnis, K.M., Thomas, S.G., Soule, J.D., Strader, L.C., Zale, J.M., Sun, T.P. and Steber, C.M. (2003) The Arabidopsis SLEEPY1 gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. *Plant Cell*, **15**, 1120–1130.

Moon, J., Parry, G. and Estelle, M. (2004) The ubiquitin–proteasome pathway and plant development. *Plant Cell*, **16**, 3181–3195.

Murfett, J., Atherton, T.L., Mou, B., Gasser, C.S. and McClure, B.A. (1994) S-RNase expressed in transgenic *Nicotiana* causes S-allele-specific pollen rejection. *Nature*, **367**, 563–566.

Nayak, S., Santiago, F.E., Jin, H., Lin, D., Schedl, T. and Kipreos, E.T. (2002) The *Caenorhabditis elegans* Skp1-related gene family: diverse functions in cell proliferation, morphogenesis, and meiosis. *Curr. Biol.* **12**, 277–287.

de Nettancourt, D. (2001) *Incompatibility and Incongruity in Wild and Cultivated Plants*. Berlin: Springer.

Petroski, M.D. and Deshaies, R.J. (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* **6**, 9–20.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C. and Genschik, P. (2003) EIN3-dependent regulation of plant ethylene hormone signaling by two arabidopsis F box proteins: EBF1 and EBF2. *Cell*, **115**, 679–689.

Qiao, H., Wang, H., Zhao, L., Zhou, J., Huang, J., Zhang, Y. and Xue, Y. (2004a) The F-box protein AhSLF-S_2 physically interacts with *S*-RNases that may be inhibited by the ubiquitin/26S proteasome pathway of protein degradation during compatible pollination in *Antirrhinum*. *Plant Cell*, **16**, 582–595.

Qiao, H., Wang, F., Zhao, L., Zhou, J., Lai, Z., Zhang, Y., Robbins, T.P. and Xue, Y. (2004b) The F-box protein AhSLF-S_2 controls the pollen function of *S*-RNase-based self-incompatibility. *Plant Cell*, **16**, 2307–2322.

Risseeuw, E.P., Daskalchuk, T.E., Banks, T.W., Liu, E., Cotelesage, J., Hellmann, H., Estelle, M., Somers, D.E. and Crosby, W.L. (2003) Protein interaction analysis of SCF ubiquitin E3 ligase subunits from Arabidopsis. *Plant J.* **34**, 753–767.

Rost, B., Yachdav, G. and Liu, J. (2004) The PredictProtein server. *Nucleic Acids Res.* **32**, W321–W326.

Russell, I.D., Grancell, A.S. and Sorger, P.K. (1999) The unstable F-box protein p58-Ctf13 forms the structural core of the CBF3 kinetochore complex. *J. Cell Biol.* **145**, 933–950.

Samach, A., Klenz, J.E., Kohalmi, S.E., Risseeuw, E., Haughn, G.W. and Crosby, W.L. (1999) The *UNUSUAL FLORAL ORGANS* gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem. *Plant J.* **20**, 433–445.

Sasaki, A., Itoh, H., Gomi, K. *et al.* (2003) Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. *Science*, **299**, 1896–1898.

Schulman, B.A., Carrano, A.C., Jeffrey, P.D., Bowen, Z., Kinnucan, E.R.E., Finnin, M.S., Elledge, S.J., Harper, J.W., Pagano, M. and Pavletich, N.P. (2000) Insights into SCF ubiquitin ligases from the structure of the Skp1–Skp2 complex. *Nature*, **408**, 381–386.

Sijacic, P., Wang, X., Skirpan, A.L., Wang, Y., Dowd, P.E., McCubbin, A.G., Huang, S. and Kao, T.-h. (2004) Identification of the pollen determinant of S-RNase-mediated self-incompatibility. *Nature*, **429**, 302–305.

Smalle, J. and Vierstra, R.D. (2004) The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **55**, 555–590.

Sonneveld, T., Tobutt, K.R., Vaughan, S.P. and Robbins, T.P. (2005) Loss of pollen-S function in two self-compatible selections of *Prunus avium* is associated with deletion/mutation of an S haplotype-specific F-box gene. *Plant Cell*, **17**, 37–51.

Tsukamoto, T., Ando, T., Watanabe, H., Marchesi, E. and Kao, T.-h. (2005) Duplication of the *S*-locus F-box gene is associated with breakdown of pollen function in an *S*-haplotype identified in a natural population of self-incompatible *Petunia axillaris*. *Plant Mol. Biol.* **57**, 141–153.

Twell, D., Yamaguchi, J., Wing, R.A., Ushiba, J. and McCormick, S. (1991) Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. *Genes Dev.* **5**, 496–507.

Ushijima, K., Sassa, H., Dandekar, A.M., Gradziel, T.M., Tao, R. and Hirano, H. (2003) Structural and transcriptional analysis of the self-incompatibility locus of almond: identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. *Plant Cell*, **15**, 771–781.

Ushijima, K., Yamane, H., Watari, A., Kakehi, E., Ikeda, K., Hauck, N.R., Iezzoni, A.F. and Tao, R. (2004) The *S* haplotype-specific F-box protein gene, *SFB*, is defective in self-compatible haplotypes of *Prunus avium* and *P. mume*. *Plant J.* **39**, 573–586.

Wang, L., Dong, L., Zhang, Y., Zhang, Y., Wu, W., Deng, X.-W. and Xue, Y. (2004) Genome-wide analysis of *S*-locus F-box-like genes in *Arabidopsis thaliana*. *Plant Mol. Biol.* **56**, 929–945.

Woo, H.R., Chung, K.M., Park, J.H., Oh, S.A., Ahn, T., Hong, S.H., Jang, S.K. and Nam, H.G. (2001) ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. *Plant Cell*, **13**, 1779–1790.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D. and Xie, D. (2002) The SCF^{COI1} ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell*, **14**, 1919–1935.

Xue, Y., Carpenter, R., Dickinson, H.G. and Coen, E.S. (1996) Origin of allelic diversity in *Antirrhinum S* locus RNases. *Plant Cell*, **8**, 805–814.

Yamanaka, A., Yada, M., Imaki, H., Koga, M., Ohshima, Y. and Nakayama, K. (2002) Multiple Skp1-related proteins in *Caenorhabditis elegans*: diverse patterns of interaction with Cullins and F-box proteins. *Curr. Biol.* **12**, 267–275.

Yasuhara, M., Mitsui, S., Hirano, H., Takanabe, R., Tokioka, Y., Ihara, N., Komatsu, A., Seki, M., Shinozaki, K. and Kiyosue, T. (2004) Identification of ASK and clock-associated proteins as molecular partners of LKP2 (LOV kelch protein 2) in *Arabidopsis*. *J. Exp. Bot.* **55**, 2015–2027.

Zhang, D., Yang, Q., Bao, W., Zhang, Y., Han, B., Xue, Y. and Cheng, Z. (2005) Molecular cytogenetic characterization of the *Antirrhinum majus* genome. *Genetics*, **169**, 325–335.

Zhao, D., Ni, W., Feng, B., Han, T., Petrasek, M.G. and Ma, H. (2003) Members of the *Arabidopsis-SKP1-like* gene family exhibit a variety of expression patterns and may play diverse roles in Arabidopsis. *Plant Physiol.* **133**, 203–217.

Zheng, N., Schulman, B.A., Song, L. *et al.* (2002) Structure of the Cul1–Rbx1–Skp1–F box Skp2 SCF ubiquitin ligase complex. *Nature*, **416**, 703–709.

Zhou, J., Wang, F., Ma, W., Zhang, Y., Han, B. and Xue, Y. (2003) Structural and transcriptional analysis of S-locus F-box genes in *Antirrhinum*. *Sex. Plant Reprod.* **16**, 165–177.

Accession numbers: DQ355479 (*AhSSK1* cDNA of class A), DQ355480 (*AhSSK1* cDNA of class B1) and DQ355481 (*AhSSK1* cDNA of class B2).

