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ORIGINAL ARTICLE

CRISPR/Cas9-mediated knockout of *PiSSK1* reveals essential role of S-locus F-box protein-containing SCF complexes in recognition of non-self S-RNases during cross-compatible pollination in self-incompatible *Petunia inflata*

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Key message **Function of *Petunia* PiSSK1.**

Abstract Self-incompatibility (SI), an inbreeding-preventing mechanism, is regulated in *Petunia inflata* by the polymorphic *S*-locus, which houses multiple pollen-specific *S-locus F-box* (*SLF*) genes and a single pistil-specific *S-RNase* gene. S_2-haplotype and S_3-haplotype possess the same 17 polymorphic *SLF* genes (named *SLF1* to *SLF17*), and each SLF protein produced in pollen is assembled into an SCF (Skp1–Cullin1–F-box) E3 ubiquitin ligase complex. A complete suite of SLF proteins is thought to collectively interact with all non-self S-RNases to mediate their ubiquitination and degradation by the 26S proteasome, allowing cross-compatible pollination. For each SCF^{SLF} complex, the Cullin1 subunit (named PiCUL1-P) and Skp1 subunit (named PiSSK1), like the F-box protein subunits (SLFs), are pollen-specific, raising the possibility that they also evolved specifically to function in SI. Here we used CRISPR/Cas9-meditated genome editing to generate frame-shift indel mutations in *PiSSK1* and examined the SI behavior of a T_0 plant (S_2S_3) with biallelic mutations in the pollen genome and two progeny plants (S_2S_2) each homozygous for one of the indel alleles and not carrying the *Cas9*-containing T-DNA. Their pollen was completely incompatible with pistils of seven otherwise-compatible *S*-genotypes, but fully compatible with pistils of an S_3S_3 transgenic plant in which production of S_3-RNase was completely suppressed by an antisense S_3-*RNase* gene, and with pistils of immature flower buds, which produce little S-RNase. These results suggest that PiSSK1 specifically functions in SI and support the hypothesis that SLF-containing SCF complexes are essential for compatible pollination.

Keywords Self-incompatibility · Skp1-like protein · PiSSK1 · CRISPR/Cas9 · S-locus F-box protein · *Petunia inflata*

Introduction

Self-incompatibility (SI) is an intraspecific reproductive barrier by which pistils reject self-pollen to prevent inbreeding and only accept non-self pollen for outcrossing (de Nettancourt 2001). SI is regulated by the highly polymorphic *S*-locus; pollen is rejected if its *S*-haplotype is identical to either *S*-haplotype of the pistil. For Solanaceae-type SI (so far found in Solanaceae, Plantaginaceae, and Rosaceae), a single *S-RNase* gene at the *S*-locus encodes the female determinant (Lee et al. 1994; Murfett et al. 1994; Broothaerts et al. 2004). For Solanaceae, Plantaginaceae, and the Maleae tribe of Rosaceae, multiple F-box genes at the *S*-locus collectively encode the male determinant. These genes were named *S-locus F-box* (*SLF*) genes in *Petunia* (Solanaceae) (Sijacic et al. 2004; Kubo et al. 2010) and *Antirrhinum* (Plantaginaceae) (Lai et al. 2002; Qiao et al. 2004), and

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named *S-haplotype-specific F-box brothers*, *SFBB*s, in Maleae (Sassa et al. 2007).

S-RNases are transported into the pollen tube in a non-*S*-haplotype-dependent manner (Luu et al. 2000; Goldraij et al. 2006), and RNase activity of S-RNases is essential for pistils to reject self-pollen (Huang et al. 1994). Therefore, non-self S-RNases (produced by pistils carrying *S*-haplotypes not present in pollen) must be detoxified inside pollen tubes to allow cross-compatibility, whereas self S-RNase (produced by pistils carrying an *S*-haplotype matching that of pollen) must not be detoxified by pollen tubes to ensure self-incompatibility. For *Petunia* (Solanaceae), the collaborative non-self recognition model was proposed to explain the different fates self and non-self S-RNases encounter inside a pollen tube (Kubo et al. 2010). The model predicts that, for a given *S*-haplotype, each SLF interacts with a subset of its non-self S-RNases to mediate their degradation via the ubiquitin–proteasome system (UPS), but none of the SLF proteins interact with their self S-RNase. Thus, a complete suite of SLF proteins is required to collectively recognize and detoxify all their non-self S-RNases to allow cross-compatible pollination.

For a conventional multi-subunit SCF (Skp1–Cullin1–F-box)-type E3 ubiquitin ligase, the F-box protein subunit interacts with its protein substrate(s), and the Cullin1 subunit serves as the scaffold, contacting Rbx1 (the subunit that binds E2 ubiquitin-conjugating enzyme) at one end, and F-box substrate at the other via an adaptor protein Skp1 (Zheng et al. 2002). To determine whether SLF proteins function as conventional F-box proteins, an SLF of *Antirrhinum hispanicum*, AhSLF-S_2, was used as bait in yeast two-hybrid screens of a pollen cDNA library, and an SLF-interacting protein, named AhSSK1 (SLF-interacting SKP1-like 1), was identified (Huang et al. 2006). *AhSSK1* is specifically expressed in pollen, but is not located at the *S*-locus. Yeast two-hybrid assays showed that AhSSK1 also interacted with another allelic variant of AhSLF, AhSLF-S_5, but unexpectedly did not interact with two other allelic variants, AhSLF-S_1 and AhSLF-S_4. Pull-down assays showed that AhSSK1 interacted with a Cullin1 protein in vitro (Huang et al. 2006). Based on sequence similarity, the AhSSK1 homolog in *Petunia hybrida*, PhSSK1, was identified, and it interacted with the N-terminal region of four SLF proteins and a Cullin1 protein of *P. hybrida* in vitro (Zhao et al. 2010). In *P. inflata*, mass spectrometric (MS) analysis of pollen proteins that co-immunoprecipitated with GFP-tagged S_2-SLF1 (S_2-SLF1:GFP) identified PiSSK1, a pollen-specific Cullin1 (named PiCUL1-P), and a conventional Rbx1 (named PiRBX1), as the subunits of the SLF-containing SCF complex (Li et al. 2014). Subsequent MS analysis of pollen proteins that co-immunoprecipitated with PiSSK1:FLAG:GFP showed that all 17 SLF proteins of S_2-haplotype and all their allelic variants in S_3-haplotype were assembled into similar SCF^{SLF} complexes (Li et al. 2016). SSK1 homologs in Maleae, including MdSSK1 of apple (*Malus domestica*) (Yuan et al. 2014; Minamikawa et al. 2014) and PbSSK1/PbSSK2 of pear (*Pyrus bretschneideri*) (Xu et al. 2013), are also pollen-specific and were found to interact with their corresponding MdSFBB or PbSLF proteins in vitro. All these results suggest that SSK1 proteins may serve as the adaptor subunit of the SCF complexes that contain SLF or SFBB proteins and that three of the four subunits of these complexes, SSK1, CUL1-P (or its homologs), and SLF (or SFBB), might have evolved to function specifically in S-RNase-based SI.

In *Petunia*, a RING-HC protein of *P. hybrida*, named PhSBP1 (S-ribonuclease binding protein), was found to interact with S_1-RNase and S_3-RNase in yeast two-hybrid screens of a pollen cDNA library (Sims and Ordanic 2001). Its ortholog in *P. inflata*, PiSBP1, was identified in yeast two-hybrid screens of a pollen cDNA library using S_2-SLF1 as bait (Hua and Kao 2006; Meng et al. 2011) and shown by in vitro binding assays to also interact with a Cullin1 (named PiCUL1-G), S-RNases, and an E2 (Hua and Kao 2006). Both SBP1 and SSK1 homologs in *M. domestica* were found to interact with an MdSFBB and a Cullin1 (MdCUL1A); however, MdSSK1 interacted with this MdSFBB more strongly than did MdSBP1 (Minamikawa et al. 2014). Thus, PiSBP1 may play the role of both Rbx1 and Skp1 in a novel E3 ubiquitin ligase complex that contains PiCUL1-G and SLF (Williams et al. 2015), which may serve as an alternative type of SLF-containing E3 ligase complex involved in detoxifying non-self S-RNases.

To address the role of SSK1 in SI, Zhao et al. (2010) used long-hairpin-RNA-mediated RNAi to suppress its expression in pollen of transgenic plants and examined the transmission of the transgene to progeny from pollination of normally compatible pistils. The RNAi lines with substantial (but not complete) reduction of PhSSK1 showed reduced transmission of the RNAi transgene to progeny from crosses with normally compatible pistils. However, analysis and interpretation of the results was complicated by the use of transgenic plants with multiple copies of the transgene, as, if not all copies were active, the percentage of pollen that inherited active copies could not be determined and thus the ratios of transgene segregation in the progeny could not be accurately predicted. That is, the severity of the phenotype observed in transgenic plants could not be precisely correlated with the degree of suppression of *PhSSK1*.

Moreover, comparative studies of knockdown and knockout mutants in model systems, including zebrafish and Arabidopsis, have shown that these two types of loss-of-function approaches did not always yield the same result (Rossi et al. 2015). For example, antisense knockdown mutants of Arabidopsis *ABP1* (*Auxin Binding Protein 1*) showed a wide range of developmental defects in root and leaf morphology, as well as altered auxin responses. However, both CRISPR/

Cas9 knockout and T-DNA insertion lines of *ABP1* (*abp1-c1* and *abp1-TD1*, respectively) didn't show any distinguishable phenotype, including showing no abnormal auxin responses (Gao et al. 2015). Also, antisense lines of Arabidopsis *MPK3* (*mitogen-activated protein kinase 3*) showed reduced stomata closure upon H_2O_2 and pathogen treatments, but its T-DNA insertion mutant (*mpk3-1*) showed normal stomata closure under these conditions (Li et al. 2017). It is not understood why contrasting results were obtained from knockdown and knockout of the same gene, but this finding suggests the importance of using both approaches to examine the function of any gene in order to obtain the most reliable information. In the case of SSK1, it is even more important, considering the complications in the interpretation of the knockdown results and considering the possibility of the involvement of an alternative type of SLF-containing E3 ligase complex.

In this work, we used CRISPR/Cas9 genome editing as the gene knockout approach to generate indel alleles of *PiSSK1* and examined the effect of a complete absence of PiSSK1 in pollen on its SI behavior. We obtained a T_0 mutant of *PiSSK1* that carried a 1-bp insertion allele and a 2-bp deletion allele in its pollen genome. Using bud-selfing to circumvent SI (Ai et al. 1990), we generated Cas9-free progeny plants homozygous for either of the two indel alleles to study the effect of each indel allele independently without the possibility of any further genome editing. We showed that a complete absence of PiSSK1 in transgenic pollen resulted in the inhibition of pollen tubes during their growth in otherwise-compatible pistils carrying non-self *S*-haplotypes and that the growth inhibition was typical of the SI response (e.g., dependent on S-RNases produced in the pistil and stoppage of most pollen tubes in the upper third segment of the pistil where most of the S-RNases are located). As none of the other nine Skp1-like proteins expressed in pollen of *P. inflata* could substitute for PiSSK1, our work conclusively demonstrated the specific and essential role of PiSSK1 in SI. The results also suggest specific interactions between PiSSK1 and a large number of diverse SLF proteins produced by pollen of many different *S*-haplotypes and support the prediction by the collaborative non-self recognition model (Kubo et al. 2010) that the SCF^{SLF} complexes in pollen are collectively required for non-self recognition of S-RNases in pollen tubes to allow cross-compatible pollination.

Materials and methods

Plant materials

All the *S*-haplotypes of *Petunia inflata* used in this work (S_2, S_3, S_5, S_{6a}, S_7, S_{11}, S_{12}, S_{13}, S_{16}, S_{24}) were from our laboratory's genetic stock (Ai et al. 1990; Wang et al. 2001; Sun and Kao 2013). *As-*S_3*/*S_3S_3 transgenic plants were obtained by bud-selfing previously generated *As-*S_3*/*S_2S_3 plants (Lee et al. 1994; Sun and Kao 2013).

Generation of Ti plasmid construct for CRISPR/Cas9-mediated knockout of *PiSSK1*

A 20-bp sequence in the first exon of *PiSSK1* (39th to 58th bp counting from the start codon ATG) was chosen as the protospacer sequence, named *PiSSK1*-Protospacer-1 (SPS1), for gene editing (Fig. 1a). The SPS1 sequence is divergent from the corresponding sequences of three previously identified pollen-expressed *Skp1-like* genes of *P. inflata*, *PiSK1*, *PiSK2,* and *PiSK3* (Hua and Kao 2006). Moreover, no off-target sites were found when this sequence, followed by any of the four protospacer adjacent motif (PAM) sequences, NGG (with N being A, G, C, or T), were used as queries to BLAST *P. inflata* S_2 and S_3 pollen transcriptomes (Williams et al. 2014), leaf transcriptome (Williams et al. 2014), and draft genome sequence (of S_{6a}-haplotype) (Bombarely et al. 2016).

We used the polycistronic tRNA-gRNA (PTG)-based CRISPR/Cas9 genome-editing system (Xie et al. 2015) to generate frame-shift indel alleles of *PiSSK1*. To generate the genome-editing Ti plasmid construct for *Agrobacterium*-mediated transformation of S_2S_3 plants, plasmid pGTR (Xie et al. 2015) was used as template to synthesize two halves of SPS1/PTG in two separate PCRs using Phusion DNA polymerase (Thermo Scientific). One reaction used *PiSSK1*-PS1-gF and L3AD5-R as primers and the other used *PiSSK1*-PS1-tR and L5AD51-F as primers (the sequences of all the primers used in this study are listed in Supplementary data Table 1). The resulting PCR fragments were ligated together using the Golden Gate Assembly method (Engler et al. 2008; Xie et al. 2015) to yield SPS1:PTG, which contained a 76-bp pre-tRNA sequence, the 20-bp SPS1, a 76-bp guide RNA (gRNA) scaffold sequence, and a 10-bp U6 transcription terminator (expanded region in Fig. 1b). The ligated fragment was further amplified using S51AD5-F and S3AD5-R primers and digested with *Fok*I (New England BioLabs). The *Fok*I-digested SPS1/PTG fragment was ligated to *Bsa*I-digested pKSE401 (deposited by the Qi-jun Chen laboratory on Addgene; Xing et al. 2014) to create the Ti plasmid construct (15,398 bp), AtU6-26P/SPS1-PTG/35SP/Cas9 (Fig. 1b), in which the *Cas9* gene was expressed by the cauliflower mosaic virus (CaMV) 35S promoter (Kay et al. 1987), and SPS1-PTG was expressed by the Arabidopsis U6-26 snoRNA promoter (AtU6-26p).

Generation of transgenic plants

The Ti plasmid construct was transformed into *Agrobacterium tumefaciens* (LBA4404) by electroporation.

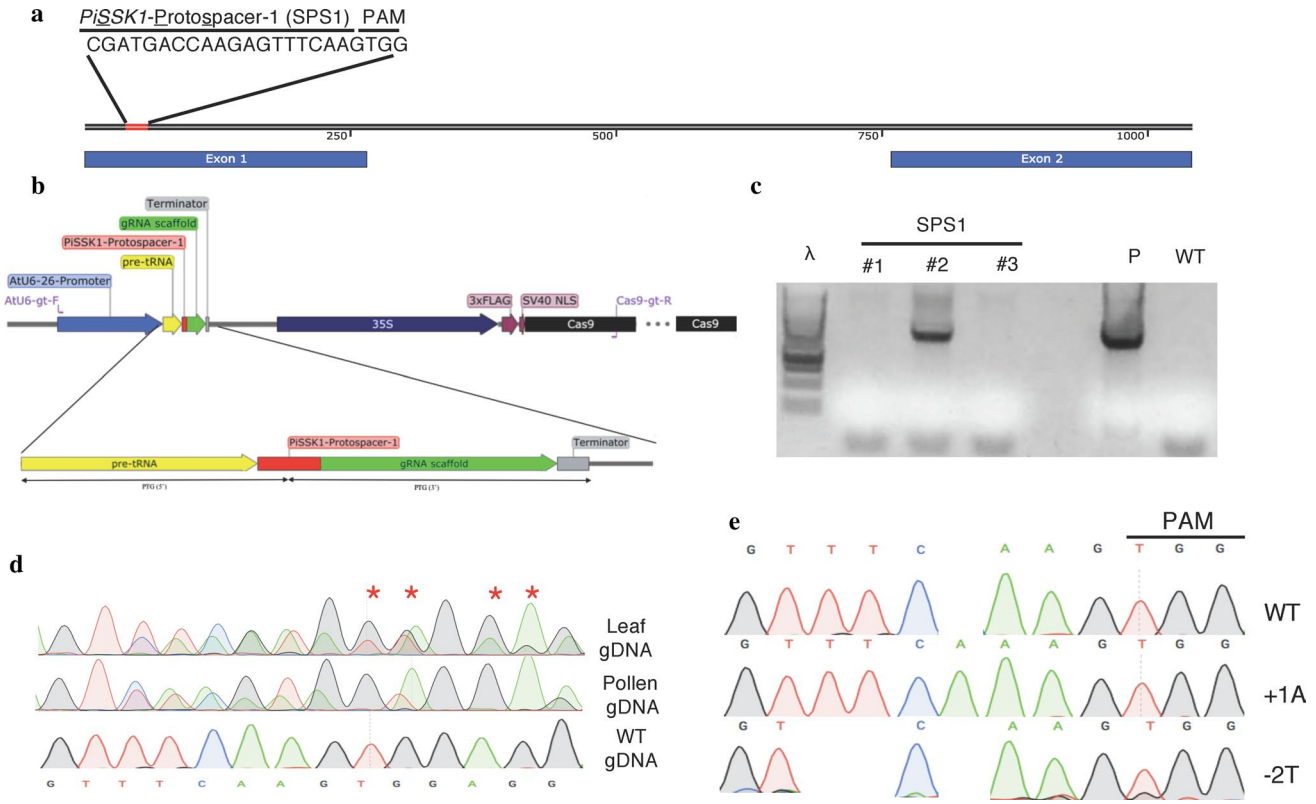


Fig. 1 CRISPR/Cas9 construct for generating *PiSSK1* knockout and sequences of resulting indel mutations in the genome-edited region. **a** Schematic of the *PiSSK1* gene and CRISPR/Cas9-targeted region. The blue blocks below the gene represent the two exons. The 20-bp sequence of the *PiSSK1*-Protospacer-1 (SPS1) spans the 39th and 58th bps (highlighted in red) of Exon 1, with the "A" of the translational start codon numbered + 1. PAM: protospacer adjacent motif. **b** Schematic of the T-DNA region containing key elements of the Ti plasmid construct used in transformation to knock out *PiSSK1*. AtU6-promoter: Arabidopsis U6-26 snoRNA promoter; terminator: U6 transcription terminator (10 consecutive T's); 35S: 35S promoter of cauliflower mosaic virus (CaMV). The SPS1-PTG fragment is shown in the expanded region. The locations of AtU6-gt-F and Cas9-gt-R primers used to amplify the *Cas9*-containing T-DNA region (~ 2.25 kb) are indicated. The sequences of these two primers are listed in Supplementary data Table 1. **c** Gel electrophoresis analysis for the presence/absence of the T-DNA in plants SPS1-1, SPS1-2, and SPS1-3 regenerated from *Agrobacterium*-mediated transformation. PCR was performed using AtU6-gt-F and Cas9-gt-R primers. λ: *Eco*RI and *Hind*III digested λ-DNA used as size markers; P: Ti plasmid DNA used in transformation (as positive control); WT: genomic DNA of a wild-type S_2S_3 plant (as negative control). **d** Sequencing chromatograms of the genome-edited region in *PiSSK1* of T_0 plant SPS1-2. Genomic DNA isolated from young leaves and pollen of SPS1-2, and from young leaves of a wild-type S_2S_3 plant, was amplified by PCR using primers *PiSSK1*-cg-F4 and *PiSSK1*-cg-R1, and the PCR products were sequenced directly. Red asterisks indicate the signature peaks for the presence of more than one indel alleles of *PiSSK1* in leaf and pollen genomic DNA of SPS1-2, as compared with the genomic DNA of wild type. The sequence of the wild-type allele is shown below the chromatograms. **e** Sequencing chromatograms of genome-edited region of *PiSSK1* in cloned PCR products amplified from leaf genomic DNA of T_0 plant SPS1-2. PCR products obtained as described in (**d**) above were cloned into a TA cloning vector, and plasmid DNA isolated from 16 randomly selected clones was sequenced. Three representative sequences are shown. WT: wild-type allele identical with *PiSSK1* in S_2S_3 wild-type plants; + 1A: an indel allele with one A inserted into the wild-type allele; − 2T: an indel allele with two T's deleted from the wild-type allele. The sequence of each allele is shown above the chromatograms

Transformation of *P. inflata* (S_2S_3) was performed as described previously (Lee et al. 1994). Genomic DNA from regenerated plants was extracted as described previously (Sun and Kao 2013). A pair of primers (*AtU6*-gt-F and Cas9-gt-R) flanking a 2.25-kb fragment, from the 3′-end of the *AtU6*-26 promoter to the 5′-end of Cas9 of the Ti plasmid construct (Fig. 1b), was used for the identification of transgenic plants.

Identification of genome-edited plants, and PCR-restriction enzyme assay of *PiSSK1* alleles

The target region in *PiSSK1* was amplified with *PiSSK1*-cg-F4 and *PiSSK1*-cg-R1 primers using Phusion DNA polymerase. The PCR products were sequenced to identify indel mutations. Overlapping peaks in the sequencing chromatograms were resolved by degenerate sequence decoding (Ma

et al. 2015). If the overlapping peaks could not be resolved, the PCR products were cloned into pGEM-T EASY vector (Promega), and positive clones were sequenced with M13 or M13R primers. The *S*-genotypes of T_0 plant SPS1-2 and its bud-selfed/bud-crossed progeny plants were determined by PCR using as primers *PiSLF2*-RT-3For and *PiSLF2*-RT-4Rev (for S_2-*SLF1*) and *PiSLF3*-Copy1For and *PiSLF3*-Copy1Rev (for S_3-*SLF1*), respectively (Li et al. 2014). For PCR-restriction enzyme assay of progeny obtained from crosses between *As*-S_3/S_3S_3 and BS-11, the PCR products, amplified by using *PiSSK1*-cg-F4 and *PiSSK1*-cg-R1 primers, were digested with *Mly*I (New England Biolabs) and then electrophoresed on a 2% agarose gel.

Pollen germination assay and seed number counting

To determine the ability of pollen to germinate in vitro, mature pollen was collected and germinated in pollen germination medium (Meng et al. 2011) for 3 h, and visualized with a Nikon Eclipse 90i epifluorescence microscope (Meng et al. 2011). Total pollen grain numbers were counted using the particle analyzing function of ImageJ (Abràmoff et al. 2004), and non-germinated pollen grains were counted manually to calculate percent of pollen germination. For each transgenic or wild-type plant examined, pollen from 5 flowers was collected and placed in separate germination media, and for every flower, 10 microscopic images were taken from different areas of the microscope slide. Seed number analysis was performed using ImageJ (Abràmoff et al. 2004).

Pollination assay and aniline blue staining of pollen tubes in pistils

Stigmas of emasculated mature flowers were manually pollinated with pollen from mature anthers. For aniline blue staining, the pollinated pistils were collected 20 h after pollination, fixed, macerated, and stained with 0.1 mg/ml aniline blue diluted with 1 mM KH_2PO_4 (1:20), according to the protocol described previously (Meng et al. 2011). The stained pistils were visualized under the DAPI-filtered UV light of a Nikon Eclipse 90i epifluorescence microscope.

Identification of *Skp1-like* genes of *Petunia inflata* and *SSK1* homologs in other Solanaceae species, and alignment of deduced amino acid sequences

The previously reported 21 Arabidopsis *Skp1-like* genes (Zhao et al. 2003) and 19 *Solanum pimpinellifolium Skp1-like* genes (Zhang et al. 2015) were aligned using ClustalW (Thompson et al. 1994) with default settings, and an HMMER (Eddy 2011) profile was generated from each alignment. The profiles were used as queries in the nhmmer searches against the CDS assembly of *P. inflata* draft genome v1.0.1 (Bombarely et al. 2016). (ftp://ftp.solgenomics.net/genomes/Petunia_inflata/annotation/Petunia_inflata_v1.0.1_CDS.fasta) HMMER hits were ranked and selected by e-value with a threshold of 1e-10, and consensus HMMER hits from two HMMER searches were compiled as *P. inflata Skp1-like* genes. Alignment of deduced amino acid sequences was performed using Clustal W (Thompson et al. 1994) with default settings. The Clustal W (alignment was visualized with BoxShade v3.2.1 https://www.ch.embnet.org/software/BOX_form.html).

Expression of the 17 *Skp1-like* genes in pollen was assessed by BLASTN search against *P. inflata* S_2 pollen and S_3 pollen transcriptomes (Williams et al. 2014). Protein secondary structure prediction was performed using PredictProtein Server (https://www.predictprotein.org/) (Rost and Liu 2003) based on PiSSK1 sequence.

For other Solanaceae *SSK1* gene identification, we used PiSSK1 protein sequence as queries in BLASTP search of protein sequence databases of several Solanaceae plant genomes (Supplementary data Table 3) on the Sol Genomics Network website (https://solgenomics.net/). The best BLASTP hits from searches against these genomes were considered as SSK1 homologs in these Solanaceae species (Supplementary data Table 3). Alignment of protein sequences was performed by ClustalW using default settings.

Accession numbers The accession numbers for the sequence data referenced in this article are as follows: S_2-SLF1 (AAS79485), S_3-SLF1 (AAS79486), S_3-RNase (AAA33727), PiSSK1 (AEE39461), PiCUL1-P (KF551593), PiRBX1 (ABB77433) from *Petunia inflata;* AhSSK1 (DQ355479) from *Antirrhinum hispanicum.* Accession numbers or sequence sources for the sequences used in Fig. 5 and Supplementary data Fig. 3 are listed in Supplementary data Table 2 and Supplementary data Table 3.

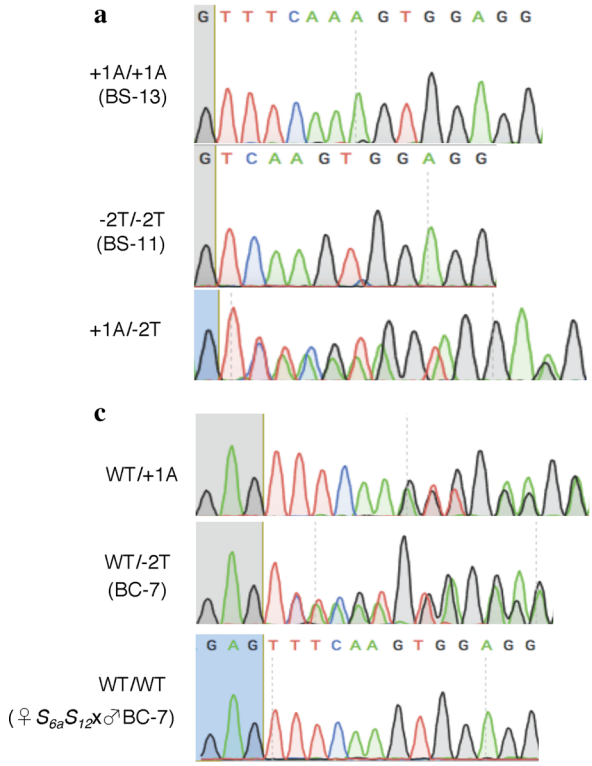
Results

Generation of two knockout alleles of *PiSSK1* by CRISPR/Cas9 genome editing

We first performed PCR on leaf genomic DNA of three plants regenerated via *Agrobacterium*-mediated transformation, using AtU6-gt-F and Cas9-gt-R as primers (Supplementary data Table 1; Fig. 1b) (the sequences of all the primers used in this study are listed in Supplementary data Table 1). A DNA fragment of ~ 2.25 kb, expected size of the amplified T-DNA region, was detected in one regenerated plant, named SPS1-2 (Fig. 1c). Genomic DNA was isolated from young leaves of this T_0 plant and amplified by PCR, using *PiSSK*1-cg-F4 and *PiSSK1*-cg-R1 as primers, to obtain DNA fragments containing the region flanking

the protospacer sequence in *PiSSK1*. Direct sequencing of the resulting PCR products revealed a mixture of sequences in this region (sequencing chromatogram-labeled "Leaf gDNA" in Fig. 1d). The PCR products were cloned, and plasmid DNA isolated from 16 randomly chosen clones was sequenced. Three different *PiSSK1* sequences in this region were obtained: wild-type (WT), a 2-bp deletion (− 2T), and a 1-bp insertion (+ 1A) (Fig. 1e). Direct sequencing of the PCR products, amplified from pollen genomic DNA using the primer pair described above, revealed the presence of only the indel alleles, − 2T and + 1A (chromatogram-labeled "Pollen gDNA" in Fig. 1d), suggesting that complete genome editing by Cas9 was achieved at late stages of plant development. Both − 2T and + 1A alleles caused frame-shift mutations and would result in truncated proteins of 36 and 35 amino acids, respectively, if they were not degraded.

As T_0 plant SPS1-2 remained self-incompatible, we used its pollen to self-pollinate pistils of immature flower buds (i.e., bud-selfing) in order to obtain *PiSSK1* knockout plants homozygous for the + 1A or − 2T allele and free of the *Cas9*-containing T-DNA. Bud-selfing can circumvent SI to set fruits, as immature flower buds produce very low levels of S-RNases insufficient to inhibit self-pollen (Ai et al. 1990). Leaf genomic DNA of each of the 33 BS (bud-selfed) plants raised was amplified by PCR using *PiSSK1*-cg-F4 and *PiSSK1*-cg-R1 as primers. Direct sequencing of the PCR products showed that all these plants were either homozygous for one of the indel alleles (− 2T/− 2T or + 1A/+ 1A) or heterozygous for the indel alleles (− 2T/+ 1A); sequencing chromatograms of three BS progeny plants representing these three *PiSSK1* genotypes are shown in Fig. 2a. Based on the sequencing results, these two indel alleles segregated into the 33 progeny plants in approximately the expected



b

PiSSK1 sequence	Expected ratio	Observed numbers (n=33)	Chi²	p-value
-2T/-2T	1	10	1.79	0.41
-2T/+1A	2	18		
+1A/+1A	1	5		

d

PiSSK1 sequence	Expected ratio	Observed number	Chi²	p-value
WT/+1A	1	7	0.53	0.47
WT/-2T	1	10		

Fig. 2 Analyses of *PiSSK1* alleles in bud-selfed (BS) and bud-crossed (BC) progenies of T_0 plant SPS1-2. **a** Sequencing chromatograms of the genome-edited region in *PiSSK1* of three BS progeny plants representing three different genotypes of *PiSSK1* alleles. Genomic DNA isolated from young leaves of 33 BS progeny plants was amplified by PCR, as described in the legend to Fig. 1d, and the resulting PCR products were directly sequenced. Three genotypes of *PiSSK1* are: + 1A/+ 1A (homozygous for the + 1A allele), + 1A/− 2T (carrying one + 1A allele and one − 2T allele) and − 2T/− 2T (homozygous for the − 2T allele). The sequences of + 1A/+ 1A and − 2T/− 2T genotypes are shown above their respective chromatograms. **b** Segregation of two indel alleles, + 1A and − 2T, of *PiSSK1* into 33 BS progeny plants of SPS1-2, based on direct sequencing of their PCR products. **c** Sequencing chromatograms in the genome-edited region of *PiSSK1* of two BC progeny plants representing two different genotypes of *PiSSK1* alleles. Sequencing was performed on PCR products of genomic DNA isolated from 17 BC progeny plants. Two genotypes of *PiSSK1* are: WT/+ 1A (carrying one wild-type allele and one + 1A allele), and WT/− 2T (carrying one wild-type allele and one − 2T allele). Sequencing chromatogram in the corresponding region of a plant carrying two wild-type alleles of *PiSSK1* is shown for comparison. This plant was obtained from the cross-indicated. **d** Segregation of two indel alleles, + 1A and − 2T, of *PiSSK1* in 17 BC progeny plants of SPS1-2, based on direct sequencing of their PCR products

1:2:1 ratio (Fig. 2b), confirming that in T_0 plant SPS1-2, − 2T and + 1A are the only two indel alleles of *PiSSK1*. PCR analysis of leaf genomic DNA of these 33 BS progeny plants, using *AtU6*-F and Cas9-gt-R as primers, revealed that 8 of them did not inherit the T-DNA (Supplementary data Fig. 1a); two of these plants, BS-11 and BS-13, were chosen for subsequent studies. The *S*-genotypes of these 33 BS progeny plants were determined by PCR using primers *PiSLF2*-RT-3For and *PiSLF2*-RT-4Rev specific to S_2-*SLF1*, and primers *PiSLF3*-Copy1For and *PiSLF3*-Copy1Rev specific to S_3-*SLF1*, and the results showed that 6 (including BS-11 and BS-13) were S_2S_2, 17 were S_2S_3, and 10 were S_3S_3 (Supplementary data Fig. 1b, c). We also used pollen of SPS1-2 to pollinate immature flower buds of wild-type S_2S_3 plants (i.e., bud-crossing). Direct sequencing of the PCR products, amplified using *PiSSK1*-cg-F4 and *PiSSK1*-cg-R1 as primers, of each of the 17 BC (bud-crossed) progeny plants raised showed that, as expected, they all contained one wild-type allele and one of the indel alleles of *PiSSK1*. Sequencing chromatograms of two BC progeny plants representing these two genotypes of *PiSSK1* are shown in Fig. 2c (labeled WT/+ 1A and WT/− 2T). Plant BC-7 (WT/− 2T) was chosen for subsequent studies. The ratio of the numbers of BC progeny plants carrying the + 1A allele or − 2T allele was approximately the expected 1:1 (Fig. 2d).

All the results described above confirm that pollen grains produced by T_0 plant SPS1-2 carried either of the two indel alleles of *PiSSK1*. That is, none of the pollen grains produced by T_0 plant SPS1-2 synthesized functional PiSSK1.

Pollen tubes carrying either indel allele of *PiSSK1* rejected by otherwise-compatible pistils

To examine the effect of the two indel mutations of *PiSSK1* on the SI behavior of transgenic pollen, we studied T_0 plant SPS1-2 (S_2S_3), and two BS plants, BS-11 (S_2S_2, − 2T/− 2T) and BS-13 (S_2S_2, + 1A/+ 1A), neither of which carried the *Cas9*-containing T-DNA (Fig. 2a; Supplementary data Fig. 1). Pollen from these three plants, as well as from wild-type S_2S_3 and S_2S_2 plants, was used to pollinate pistils of wild-type S_3S_3, $S_{6a}S_{12}$, S_7S_{16}, S_3S_{13}, S_3S_{24}, $S_{11}S_{11}$, and S_5S_5 plants. At least three pollinations were performed for each cross. As expected, pollen of wild-type S_2S_3 and S_2S_2 plants was compatible with pistils of all these seven *S*-genotypes (each carrying at least one *S*-haplotype different from S_2 or S_3); however, no fruit was set when pollen of SPS1-2, BS-11, or BS-13 was used to pollinate pistils of these seven otherwise-compatible *S*-genotypes (Table 1).

To assess whether the inability to set fruit was due to inhibition of pollen tube growth in the pistil, we used aniline blue to stain pollen tubes in wild-type S_3S_3 and $S_{6a}S_{12}$ pistils 20 h after separate pollination by pollen of SPS1-2, BS-11, BS-13, and wild-type S_2S_3, S_2S_2, S_3S_3 and $S_{6a}S_{12}$ plants (Fig. 3). In all cases, growth of most pollen tubes carrying − 2T and/or + 1A allele of *PiSSK*1 stopped in the upper third segment of the pistil, similar to the "incompatible" controls where a wild-type $S_{6a}S_{12}$ plant was self-pollinated. In contrast, the majority of pollen tubes from the "compatible" controls, i.e., pollination of $S_{6a}S_{12}$ pistils with pollen from wild-type S_2S_2 and S_2S_3 plants, grew to the bottom of the pistil. To verify that pollen tubes carrying one of the indel alleles were unable to grow through the pistil to effect fertilization, we used pollen from BC-7 (Fig. 2c) to pollinate pistils of wild-type $S_{6a}S_{12}$ plants. BC-7 (S_2S_3) carried the wild-type allele and the − 2T indel allele of *PiSSK1*, but did not carry the *Cas9*-containing T-DNA. Genomic DNA of 20 progeny plants raised was amplified by PCR using *PiSSK1*-cg-F4 and *PiSSK1*-cg-R1 as primers, and the resulting PCR products were sequenced. All were homozygous for the wild-type allele; the sequencing chromatogram of one representative plant, labeled WT/WT, is shown in Fig. 2c. This result suggests that only pollen tubes carrying the wild-type allele of *PiSSK1* can effect fertilization.

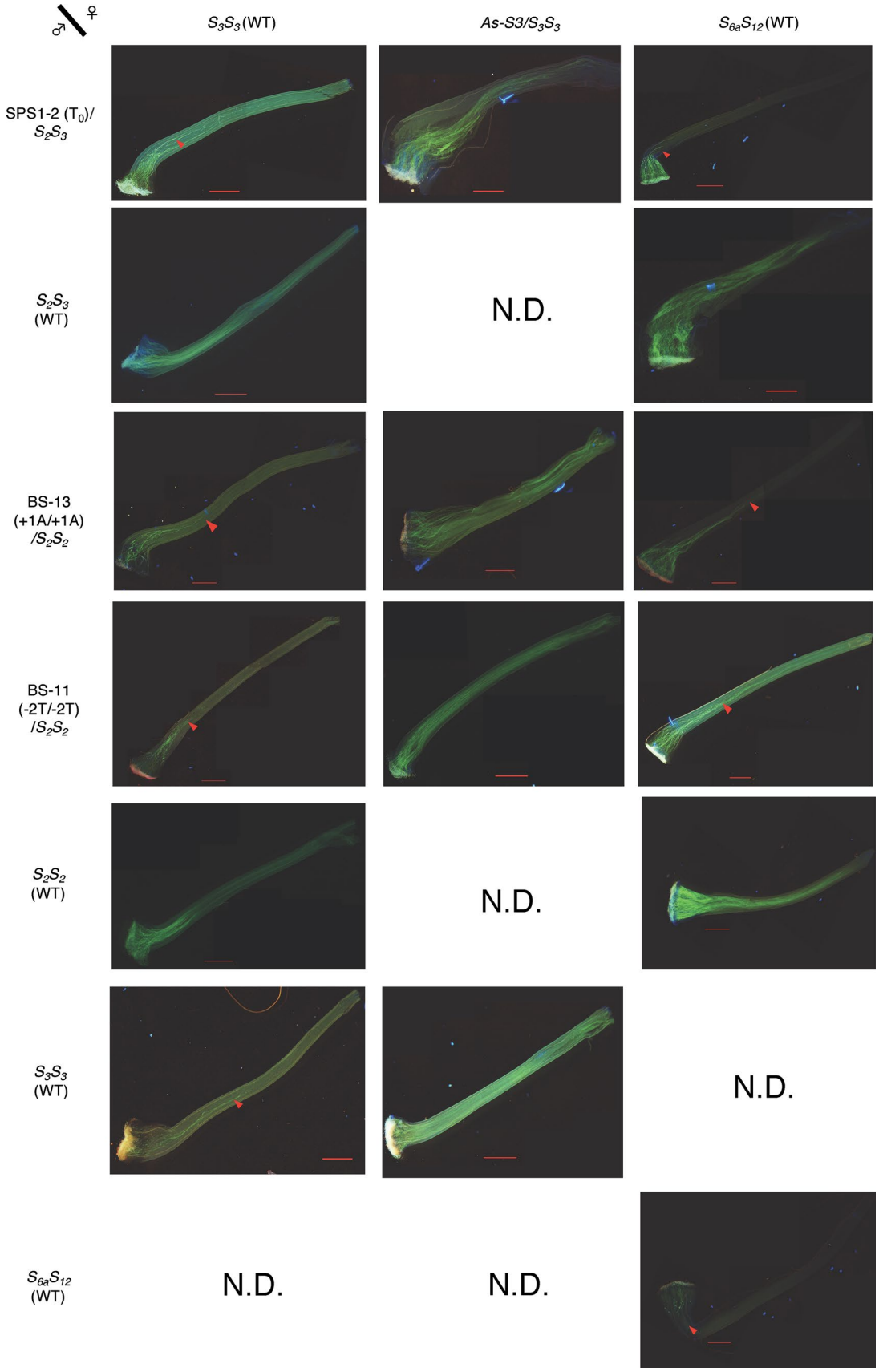
Table 1 Summary of pollination results obtained in this study

♀	♂				
	SPS1-2 (T_0)	S_2S_3	BS-11 (− 2T/− 2T)	BS-13 (+ 1A/+ 1A)	S_2S_2
S_3S_3	− (6)	+	− (7)	− (5)	+
$S_{6a}S_{12}$	− (5)	+	− (7)	− (5)	+
S_7S_{16}	− (6)	+	− (5)	− (5)	+
S_3S_{13}	− (5)	+	− (5)	− (5)	+
S_3S_{24}	− (5)	+	− (5)	− (3)	+
$S_{11}S_{11}$	− (5)	+	− (5)	− (5)	+
S_5S_5	− (5)	+	− (5)	− (5)	+
As-S_3/S_3S_3	+ (5/5)	+	+ (3/3)	+ (2/2)	+
Self bud	+ (8/10)	+	+ (3/5)	+ (4/6)	+
S_2S_3 bud	+ (6/8)	+	+	+	N.D.

"–": incompatible pollination, with each number in parentheses indicating number of replicates. "+": compatible pollination, with each ratio in parentheses indicating number of pollinations setting fruits over number of pollinations performed. N.D.: not determined. *As*-S_3/S_3S_3: a self-compatible transgenic plant not producing S_3-RNase in the pistil. "Self bud" and "S_2S_3 bud": pollen was used to pollinate pistils of immature buds of the same plant and a wild-type S_2S_3 plant, respectively

No effect of indel mutations of *PiSSK1* on pollen viability or pollen tube growth in vitro

To rule out the possibility that knocking out *PiSSK1* might reduce pollen viability or affect pollen tube growth, we


♂ \ ♀
S_3S_3 (WT)
As-S3/S_3S_3
$S_{6a}S_{12}$ (WT)
SPS1-2 (T_0)/ S_2S_3
S_2S_3 (WT)
N.D.
BS-13 (+1A/+1A) /S_2S_2
BS-11 (-2T/-2T) /S_2S_2
S_2S_2 (WT)
N.D.
S_3S_3 (WT)
N.D.
$S_{6a}S_{12}$ (WT)
N.D.
N.D.

◂**Fig. 3** Fluorescent microscopic images of aniline blue-stained pollen tubes in pistils 20 h after manual pollination. In cases where pollen tube growth is stopped, the red arrow in each pistil indicates where growth of most pollen tubes stopped. N.D.: not determined. Scale bar = 1 mm

examined in vitro germination of pollen from SPS1-2, BS-11, BS-13, and wild-type S_2S_2 and S_2S_3 plants (Fig. 4a–e); no significant difference in the ability of the pollen from these plants to germinate was observed (Fig. 4f). These results suggest that if there are any non-SLF F-box proteins required for pollen development/function, their function is not affected by the loss-of-function of *PiSSK1*, as otherwise we would expect to observe pollen development or pollen function phenotypes in the *PiSSK1* knockout plants.

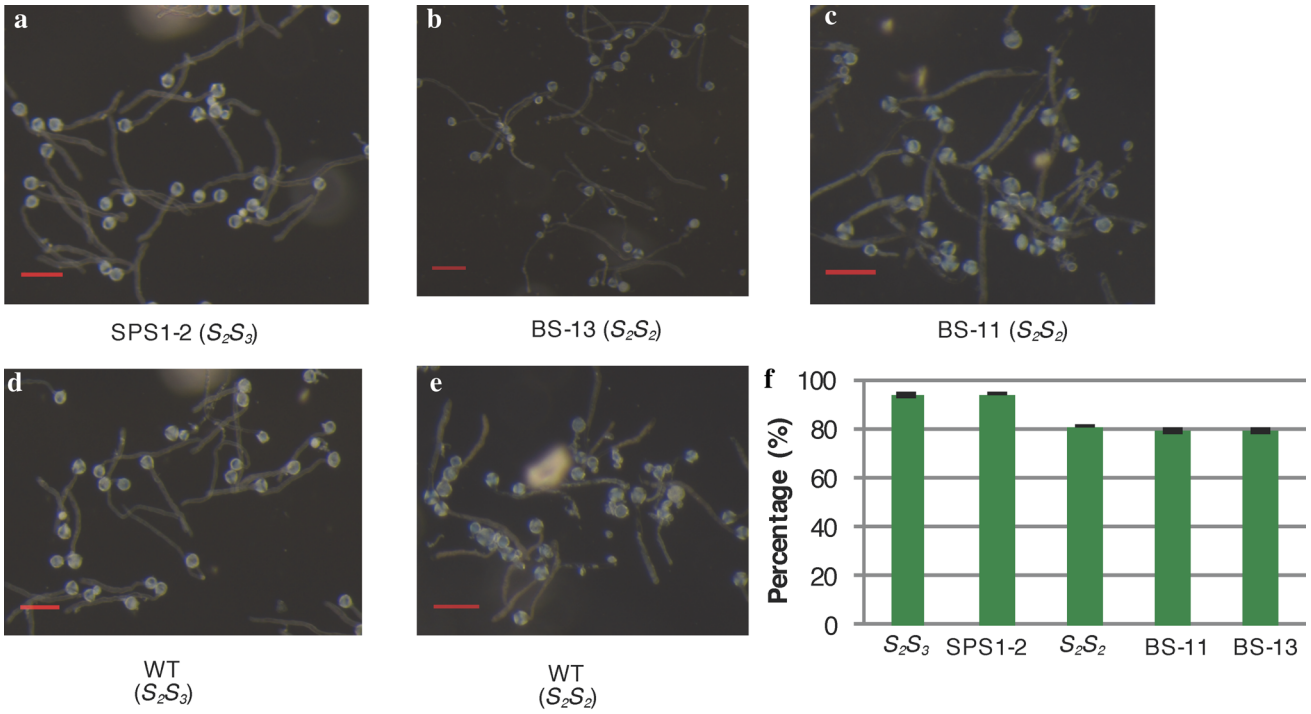
Rejection of *PiSSK1* knockout pollen by otherwise-compatible pistils dependent on S-RNases produced in the pistil

We next examined whether rejection of pollen of SPS1-2, BS-11, and BS-13 by pistils of otherwise-compatible *S*-genotypes during tube growth (Table 1; Fig. 3) was dependent on the S-RNases produced in the pistil. We used pollen of these three plants and a wild-type S_3S_3 plant to pollinate a self-compatible transgenic plant, *As-*S_3/S_3S_3 (*As-*S_3), whose S_3-RNase production in the pistil was completely suppressed by an antisense S_3-*RNase* transgene (Lee et al. 1994; Sun and Kao 2013). Aniline blue staining of pollinated pistils showed that the majority of the pollen tubes of SPS1-2, BS-11, and BS-13 grew to the bottom of the pistil, similar to the pollination by pollen of the wild-type S_3S_3 plant (Fig. 3). Moreover, pollination of *As-*S_3 with pollen of SPS1-2, BS-11, and BS-13 yielded fruits, similar to pollination with pollen of wild-type S_2S_2 and S_2S_3 plants (Table 1), and the seed numbers of the fruits produced were comparable (Fig. 4g).

As transgenic plant *As-*S_3 was self-compatible, the observed seed set from pollination by pollen of SPS1-2, BS-11, and BS-13 could have entirely resulted from self-pollination of *As-*S_3. To examine this possibility, we raised 16 progeny plants from pollination of *As-*S_3 pistils by pollen of BS-11 (− 2T/− 2T) and determined the *PiSSK1* alleles they carried by a PCR-restriction enzyme assay. The PCR product of the − 2T allele, but not the wild-type allele, of *PiSSK1* could be digested by restriction enzyme *Mly*I, yielding two fragments of 155 and 160 bp. The gel electrophoresis results showed that 5 (#1, 5, 7, 11, 13) of the 16 progeny plants had the same *MlyI*-digestion pattern as that of the wild-type plant, whereas the rest had the same *MlyI*-digestion pattern as that of BC-7 (Supplementary data Fig. 2a). We next performed PCR, using primers specific to S_2-*SLF1*, to determine whether these 16 progeny plants carried S_2-haplotype (Supplementary data Fig. 2b). Absence of the PCR fragment for the five progeny plants that only carried the wild-type allele of *PiSSK1* suggests that they were S_3S_3 and most likely derived from inadvertent dropping of self-pollen onto the pistil of self-compatible *As-*S_3 prior to, or during, manual pollination by pollen from BS-11. The PCR fragment was detected in the other 11 progeny plants that carried one wild-type allele and one − 2T allele of *PiSSK1*, confirming that they were derived from pollination of *As-*S_3/S_3S_3 pistils by pollen of BS-11 (S_2S_2), with the wild-type allele inherited from *As-*S_3 and the − 2T allele inherited from BS-11. Thus, absence of S_3-RNase in the pistil of *As-*S_3 allowed pollen tubes carrying the − 2T allele to grow through the pistil to effect fertilization. This is consistent with the findings that bud-selfing of SPS1-2, BS-11, and BS-13, and bud-crossing of SPS1-2 with a wild-type S_2S_3 plant, all resulted in fruits with seed numbers comparable to those of fruits obtained from bud-selfing of wild-type S_2S_2 and S_2S_3 plants (Fig. 4g). All these results also suggest that knocking out *PiSSK1* does not affect pollen development or pollen function, as pollen tubes carrying mutated *PiSSK1* were able to grow through the pistil in the absence of S-RNases, or in the presence of insufficient amounts of S-RNase, and the resulting fertilization gave rise to normal progeny.

A 13-amino acid sequence unique to PiSSK1 and SSK1 orthologs in other solanaceous species, but not present in 16 other Skp1-like proteins of *Petunia inflata*

As a first step toward studying the biochemical basis for the specific interaction between PiSSK1 and SLF proteins, we compared the amino acid sequences of PiSSK1 and all other Skp1-like proteins of *P. inflata* to see whether PiSSK1 possesses any unique sequence feature. We used the sequences of *Skp1-like* genes of Arabidopsis (Zhao et al. 2003) and *Solanum pimpinellifolium* (a wild species of tomato) (Zhang et al. 2015) to search the *P. inflata* draft genome (Bombarely et al. 2016). A total of 17 *Skp1-like* genes were identified, and 10 of them were found in the S_2 and/or S_3 pollen transcriptomes (Williams et al. 2014), including *PiSSK1* and three previously identified *Skp1-like* genes, *PiSK1*, *PiSK2,* and *PiSK3* (Hua and Kao 2006) (Supplementary data Table 2). Alignment of the deduced amino acid sequences of PiSSK1 and 16 other Skp1-like proteins revealed a stretch of 13-amino acids unique to PiSSK1 (highlighted in red in Fig. 5), which resides in the predicted second and third helices of the secondary



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♀ \ ♂	SPS1-2 (T_0)	BS-11 (-2T/-2T)	BS-13 (+1A/+1A)	S_2S_3	S_2S_2
As-S3/S3S3	111.6±17.7 n=5	127±3.51 n=3	99.0±6.0 n=2	135±28.2 n=3	107.3±18.9 n=3
Self bud	125.1±10.9 n=8	66.7±14.2 n=3	53.3±16.2 n=4	109.5±78.9 n=4	70.2±10.6 n=5
S_2S_3 bud	43±26.3 n=6	N.D.	N.D.	N.D.	N.D.

Fig. 4 In vitro germination of pollen from T_0 plant SPS1-2 and two bud-selfed plants, BS-11 and BS-13, and seed set from their bud-selfing, bud-crosses, and from pollination of a self-compatible transgenic plants that did not produce S_3-RNase. **a**–**e** Representative dark field microscopic images of in vitro germinated pollen tubes of T_0 plant SPS1-2 (**a**), BS-11 (**b**), BS-13 (**c**), and wild-type S_2S_3 (**d**) and S_2S_2 (**e**) plants. Scale bar = 0.2 mm. **f** Percent pollen tube germination of the plants used in the *in vitro* germination assay. Average percentage of pollen tube germination of five flowers, each with ten microscopic images taken under dark field, was calculated and shown as mean ± standard error % in the graph. **g** Seed set from compatible pollination. *As-S3/S3S3*, a self-compatible transgenic plant; self bud: self-pollination at immature bud stages; S_2S_3 bud: pollination of pistils of immature buds of wild-type S_2S_3 plants. "*n*" is the number of fruits analyzed, and seed numbers are shown as mean ± standard error

structure of PiSSK1. To examine whether this unique sequence feature is conserved among PiSSK1 orthologs in other Solanaceae species, we identified PiSSK1 orthologs from the genome sequence databases of 10 other Solanaceae species (Supplementary data Table 3). Alignment of the deduced amino acid sequences of these Solanaceae SSK1 proteins showed that all shared this additional stretch of sequence with the length ranging from 11 to 17 amino acids (Supplementary data Fig. 3a). Interestingly, alignment of the deduced amino acid sequences of PiSSK1, PiSK1, and AhSSK1 showed that AhSSK1, like the other Skp1-like proteins of *P. inflata*, did not have this sequence feature (Supplementary data Fig. 3b).

Discussion

The finding that multiple paralogous S-locus F-box (SLF) proteins collectively specify the male determinant of S-RNase-based SI possessed by *Petunia* and several other species led to the hypothesis that SLF proteins are involved in ubiquitin-mediated degradation of the S-RNases with which each SLF protein interacts (Kubo et al. 2010; Williams et al. 2015). This hypothesis has subsequently been confirmed by showing that S_7-SLF2 of *P. hybrida*, and all 17 polymorphic SLF proteins produced in S_2 and S_3 pollen of

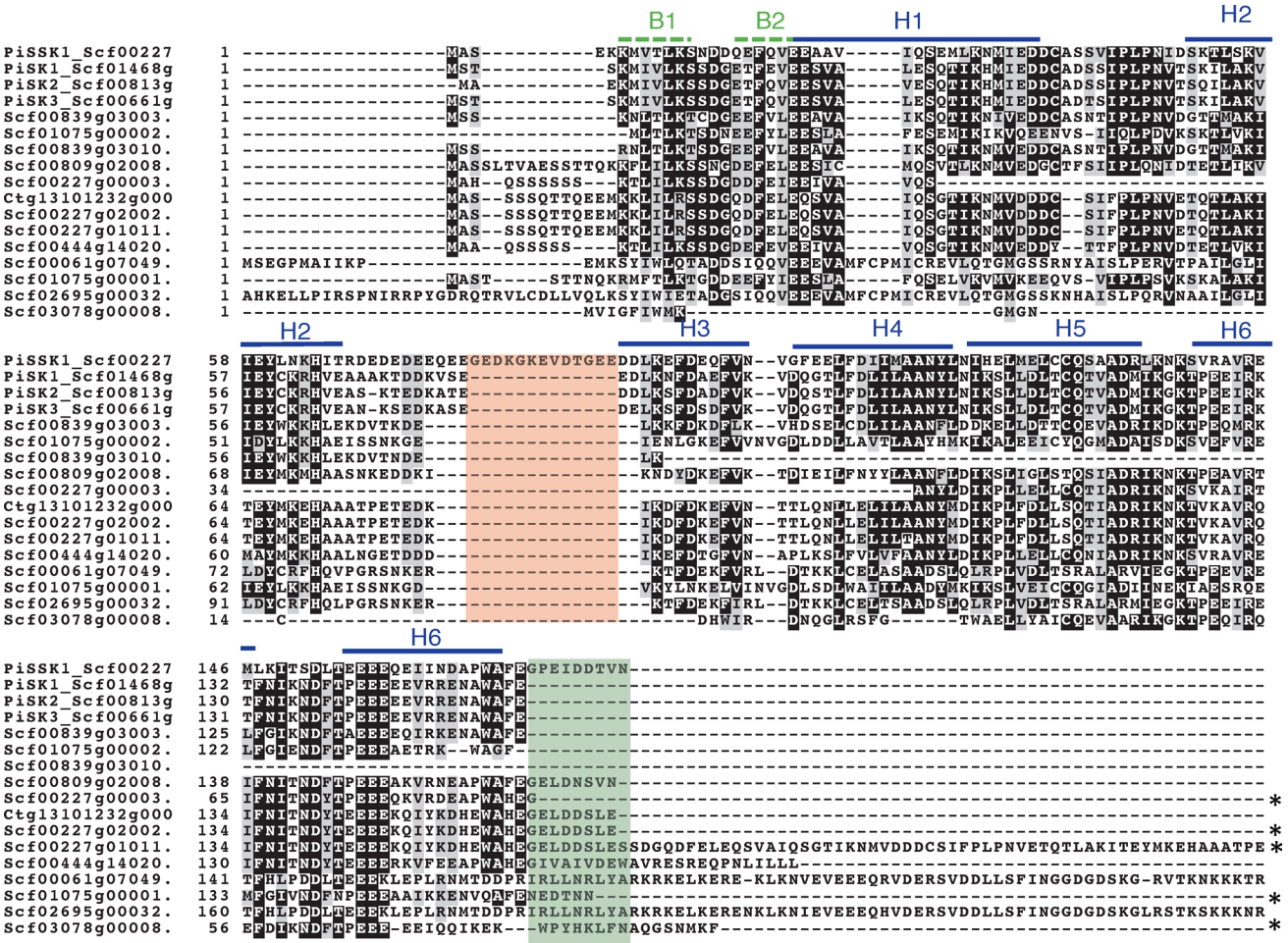

Fig. 5 Alignment of deduced amino acid sequences of PiSSK1 and 16 other Skp1-like proteins of *Petunia inflata*. The 13-amino acid stretch unique to PiSSK1 is highlighted in red. The C-terminal 9 amino acids previously identified as a unique feature of PhSSK1 (Zhao et al. 2010) are highlighted in green. The extended C-terminal sequences of five type-II Skp1-like proteins (Kong et al. 2007), indicated with asterisks, are not shown. The secondary structural features predicted for PiSSK1 are shown above the aligned sequences. All the accession numbers of Skp1-like proteins extracted from the *Petunia inflata* draft genome (Bombarely et al. 2016) are listed in Supplementary data Table 2

P. inflata, co-immunoprecipitated with proteins homologous to Skp1 and Cullin1, respectively, two of the subunits of a canonical SCF E3-ubiquitin ligase complex (Li et al. 2014; Entani et al. 2014; Li et al. 2016). Both the Skp1 homolog, SSK1, and the Cullin1 homolog, CUL1-P, are pollen specific, raising the possibility that, like the F-box protein subunit, these two other subunits of the SCF^{SLF} complex also specifically function in SI.

In this work, we used CRISPR/Cas9 genome-editing technology to address the question of whether PiSSK1 is required for cross-compatible pollination. We designed the gRNA to allow Cas9 to specifically target a 20-bp coding sequence for amino acids near the N-terminal region of PiSSK1 (Fig. 1a) and identified a transgenic plant, SPS1-2, that carried two indel alleles of *PiSSK1*, one with a deletion of 2 bp (− 2T) and the other with an insertion of 1 bp (+ 1A). From sequencing the PCR products of the genome-edited region of leaf and pollen genomic DNA, and subsequent sequencing of TA clones of the PCR products of leaf genomic DNA, as well as from segregation of these two indel alleles in bud-selfed (BS) progeny and bud-crossed (BC) progeny of SPS1-2 (Figs. 1d, e, 2), we concluded that pollen produced by this T_0 plant carried either the − 2T allele or the + 1A allele of *PiSSK1*. Thus, *PiSSK1* was knocked out in all pollen grains produced by SPS1-2 and by its progeny homozygous for either indel allele. When using RNAi to suppress the expression of a pollen-specific gene, one would normally study transgenic plants carrying one copy of the transgene, so as to more precisely assess the phenotypic effect of the transgene on the transgenic pollen. In this case, 50% of the pollen produced would be wild type, and thus the maximum level of suppression of the transgene

(e.g., measured by quantitative PCR of the transcripts produced by pollen collected from one such transgenic plant) would be 50%. With this high background of the transcript contributed by the wild-type pollen, it is often difficult to precisely assess the degree of suppression in each transgenic plant and then to correlate the degree of suppression with the severity of the observed phenotype. Although using transgenic plants carrying multiple copies of the transgene would in theory increase the level of suppression, this would complicate interpretation of the effect of suppression, as it is difficult, if not impossible, to determine whether all copies, or only a certain number of the copies, of the transgene are active.

Moreover, the CRISPR/Cas9 genome-editing technology offers another advantage. After genome-edited transgenic plants are identified, one could use selfing or crosses to segregate out the inserted T-DNA that carries *Cas9* and study those progeny plants that are free of *Cas9*. In this way, no further genome editing will occur to complicate the phenotypic analysis. In our case, we performed bud-selfing on SPS1-2 and from analysis of 33 progeny plants identified 8 plants that did not inherit the *Cas9*-containing T-DNA (Supplementary data Fig. 1a). Bud-selfing also allowed us to generate plants that were homozygous for either the − 2T allele or the + 1A allele of *PiSSK1* (Fig. 2b), so that we could examine the effect of each indel allele independently. In this study, we performed comprehensive analysis of the SI behavior of SPS1-2 (S_2S_3, + 1A/− 2T) and two of its bud-selfed (BS) progeny plants, BS-11 (S_2S_2, − 2T/− 2T) and BS-13 (S_2S_2, + 1A/+ 1A). We selected wild-type plants of seven *S*-genotypes (collectively carrying nine different *S*-haplotypes) to use as female in genetic crosses, as they did not carry S_2-haplotype and would thus normally be compatible with S_2 pollen produced by SPS1-2, BS-11, and BS-13. Lack of fruit set (Table 1) suggests that pollen of SPS1-2, BS-11, and BS-13, not producing any functional PiSSK1, cannot effect fertilization of these otherwise-cross-compatible female plants. We further obtained three lines of evidence to show that the failure of fertilization in all these crosses was due to the presence of the S-RNases produced by the pistils of these female plants. First, aniline blue staining of pollinated pistils of S_3S_3 and $S_{6a}S_{12}$ showed that, characteristic of the arrest of incompatible pollen tubes, the majority of pollen tubes did not grow beyond the upper third segment of the pistil (Fig. 3), where most of the S-RNases in the pistil are present (Ai et al. 1990). Second, normal-size fruits were obtained from both bud-selfing of SPS1-2, BS-11, and BS-13, and from bud-crosses of SPS1-2 with wild-type pistils of S_2S_3 genotype (Table 1; Fig. 4g), consistent with the well-known fact that pistils of immature buds produce little S-RNase and cannot reject self pollen (Ai et al. 1990). Third, pollen of SPS1-2, BS-11, and BS-13 was completely compatible with pistils of a self-compatible transgenic plant, *As-*S_3*/*S_3S_3, which did not produce any S_3-RNase in the pistil (Table 1), and all pollinations set normal-size fruits (Fig. 4g). Analysis of the *PiSSK1* alleles carried by the progeny obtained from pollination by BS-11 showed that two-thirds of them indeed resulted from fertilization by pollen of BS-11 (the rest from inadvertent self-pollination of *As-*S_3*/*S_3S_3) (Supplementary data Fig. 2).

All the results taken together strongly support (1) the finding by co-immunoprecipitation and mass spectrometry that PiSSK1 is the Skp1 subunit of all SCF^{SLF} complexes (Li et al. 2016) and (2) the prediction by the collaborative non-self recognition model that SCF^{SLF} complexes are collectively required to mediate ubiquitination and degradation of all non-self S-RNases to ensure cross-compatible pollination in a species possessing S-RNase-based SI (Kubo et al. 2010).

The human genome has only one *Skp1* gene (Schulman et al. 2000), but plant genomes have multiple diverse *Skp1-like* genes. For example, Arabidopsis and rice genomes possess 21 and 28 *Skp1-like* genes, respectively (Kong et al. 2004, 2007). To date, the physiological functions of most of the plant Skp1-like proteins remain uncharacterized. The best characterized plant Skp1-like protein, ASK1 in Arabidopsis, has been shown to be assembled into diverse SCF complexes, including SCF^{TIR1} in auxin signaling and SCF^{COI} in jasmonic acid signaling (Gray et al. 2001; Xu et al. 2002), and not surprisingly, *ask1* mutants showed a pleiotropic phenotype (Zhao et al. 1999). However, some Arabidopsis Skp1-like proteins appear to have redundant functions. For example, *ask2* mutants showed no phenotype while *ask1ask2* double mutants had severe developmental defects during the seedling stage (Liu et al. 2004). RT-PCR and yeast two-hybrid or bimolecular fluorescence complementation (BiFC) assays showed that some ASK proteins were only produced in certain tissues or organs, and others, such as ASK5 and ASK16, only interacted with a subset of F-box proteins (Gagne et al. 2002; Risseeuw et al. 2003; Kong et al. 2004; Dezfulian et al. 2012). To the best of our knowledge, so far no single Skp1-like protein of plants has been shown to be exclusively involved in one particular biological process, or to specifically interact with a certain class/type of F-box protein. The SI phenotype of the *PiSSK1* knockout plants studied here suggests that PiSSK1 is an unusual Skp1-like protein as it may function exclusively in one biological process, SI, and no other Skp1-like proteins produced in pollen can substitute for PiSSK1 in the assembly of SCF^{SLF} complexes.

The indispensable role of PiSSK1 in the assembly of SCF^{SLF} complexes also suggests that a single PiSSK1 must interact with all SLF proteins in order to allow them to function in SI. For example, 32 *S*-haplotypes have been reported

for *Petunia* so far (Sims and Robbins 2009; Wang and Kao 2012), and if we assume that each *S*-haplotype has a suite of 17 polymorphic SLF proteins (as has been found for S_2 and S_3), PiSSK1 would have to interact with 544 divergent SLF proteins (e.g., the amino acid identity between 17 paralogous SLF proteins of S_2 pollen ranges from 45 to 88%; Williams et al. 2014). We previously used transcriptome analysis to identify 179 additional F-box proteins in S_2 and S_3 pollen *P. inflata* (Williams et al. 2014), but our Co-IP/MS experiments showed that only one pair of SLF-like proteins and two pairs of non-SLF F-box proteins could interact with PiSSK1 (Li et al. 2016). This is consistent with our observation in this study that pollen development and pollen function in fertilization were not affected in the *PiSSK1* knockout plants and that *PiSSK1* knockout pollen was able to fertilize pistils that produced very low levels of S-RNases. It would be of interest to study the biochemical basis for the specific interaction between PiSSK1 and SLF proteins.

As a first step to address the specific interaction between PiSSK1 and SLF proteins, we compared the deduced amino acid sequences of PiSSK1 and 16 other Skp1 proteins predicted by the genome sequence of *P. inflata*, and identified a 13-amino acid stretch specific to PiSSK1. The secondary structure of PiSSK1, predicted based on the known structures of Skp1-like proteins, revealed that this 13-amino acid stretch lies between the 2nd and 3rd α-helices as part of a flexible "acidic loop" (Fig. 5). Enrichment of this region with aspartic acid and glutamic acid residues suggests its structural flexibility. This acidic loop has been found in the structures of most Skp1-like proteins in eukaryotes (Zheng et al. 2002). It has been proposed that the flexible acidic loop in human Skp1 could clash with F-box protein-exchange-factor Cand1 (Cullin-associated and neddylation-dissociated protein 1) and facilitate exchange of multiple Skp1–F-box complexes with the Cul1–Rbx1 scaffold (Pierce et al. 2013). Since all SLF proteins use the same Cul1–Rbx1 scaffold (PiCUL1P–PiRBX1 in *P. inflata*; Li et al. 2014, 2016), it is possible that this extended loop region unique to PiSSK1 functions to promote the exchange between different SLF proteins in order to maximize the assembly of those SLF proteins that are capable of interacting with the non-self S-RNases to be detoxified during pollen tube growth in the pistil of a particular *S*-genotype. As this extended loop region is absent in AhSSK1 (Supplementary data Fig. 3), when Zhao et al. (2010) compared the deduced amino acid sequences of AhSSK1, PhSSK1, and 14 Skp1-like proteins from Arabidopsis (2), *Antirrhinum* (3), and *Petunia* (9), they identified a C-terminal extension of 7 and 9 amino acids, beyond the conventional "WAFE" motif present in most Skp1-like proteins, as a unique feature of AhSSK1 and PhSSK1, respectively (the region highlighted in green in Fig. 5). However, 9 of the 16 other Skp1-like proteins from *P. inflata* included in the alignment with PiSSK1 also have this C-terminal extension (Fig. 5). Further study of the role, if any, of this C-terminal extension and the 13-amino acid region of SSK1 would help shed light on how the interaction specificity between a single SSK1 protein and a large number of diverse SLF proteins was established and how it has been maintained during the course of evolution.

This work has also ruled out the involvement of any other alternative type of SLF-containing SCF ubiquitin E3 ligase complexes in SI, such as a possible type of PiSBP1-forming SCF-like complex. In vitro, PiSBP1 catalyzed ubiquitination of S_3-RNase in conjunction with E1 ubiquitin-activating enzyme and E2 ubiquitin-conjugating enzyme (Hua and Kao 2006), so it has been proposed to be involved in regulating the basal level of SLF proteins in pollen, as a safety mechanism to ensure that all non-self S-RNases are cleared from compatible pollen tubes (Williams et al. 2015). It is interesting to note that a recent study on a *Caenorhabditis elegans* Skp1-related protein, SUP-36, showed that SUP-36 functioned as an inhibitor of normal SCF complexes and could be coordinately ubiquitinated and degraded by two E2/E3 pairs: UBCH7 (UBC-18) and RBR E3 HHARI (ARI-1), and CDC34 (UBC-3) and a canonical SCF E3 ligase complex (Dove et al. 2017). As PiSBP1 has a partial Skp1-related property, it might also be subject to similar regulation as is SUP-36.

Establishing and maintaining SI requires not only that self-pollination be incompatible, but also that cross-pollination be compatible. After establishment of the system involving a minimum of three *S*-haplotypes, selection pressure would favor generation of new haplotypes, as pollen carrying a new haplotype would successfully pollinate all pistils carrying existing haplotypes. During the evolution of SI in *Petunia*, all allelic variants of any newly emerged SLF would have to be able to interact with PiSSK1 in order to maintain compatibility between plants of different *S*-genotypes. Considering the essential role of PiSSK1 in SI, one might expect gene redundancy to safeguard the lethal effect its loss-of-function mutation has on pollen. For a plant carrying a wild-type allele and a loss-of-function allele, those pollen grains (50% of total) carrying the wild-type allele will be transmitted to the progeny through cross-pollination, but those carrying the mutant allele will be rejected by pistils of any *S*-genotype. Thus, the mutant allele will not spread in a self-incompatible natural population through pollen. However, as PiSSK1 is only required for pollen SI function, the pistil of this plant can be pollinated by compatible pollen from other plants, the mutant allele will still be retained in the population.

Interestingly, CUL1-P, the Cullin1 subunit of SCF^{SLF} complexes in *Petunia*, is also pollen-specific (Li et al. 2014, 2016), and PhCUL1-P (CUL1-P of *P. hybrida*) has been shown by artificial microRNA suppression to be essential for cross-compatible pollination in SI (Kubo et al. 2016). It

would be interesting to use CRISPR/Cas9-mediated genome editing to knock out *PiCUL1-P* in *P. inflata* to see whether PiCUL1-P specifically functions in SI and whether any other pollen-expressed Cullin1 might be able to substitute for PiCUL1-P in its absence.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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