Synthetic mRNA was injected into the yolk of embryos previously injected with morpholino. The following plasmids were linearized, and sense-strand-capped mRNA was synthesized: pCS2 + zebrafish LIV1 (NotI, SP6), pCS2 + zebrafish STAT3 (NotI, SP6)[10], and pCS2 + mouse Snail (NotI, SP6)[14]. The cDNA sequence of full-length mouse Snail (1–264)[20] and C-terminus-deleted mouse Snail (1–151)[20] was amplified by high-fidelity PCR using specific oligonucleotides primers that created an EcoRI site at the 3′ end. The resulting PCR product was cloned into a unique EcoRI site located upstream of the ATG of the GFP gene in the pCS2 expression plasmid.

Cell culture, RNAi and RT–PCR

Mouse proB cell lines Baf/B03 stably expressing human G-CSFR-gp130 chimaeric receptors were maintained as described[17]. Cells were starved with factor-free medium for 6 h followed by stimulation with medium containing 100 ng ml^{-1} of human G-CSF for the indicated time. Human prostate-cancer cell line DU145 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The small interfering RNA (siRNA) for human STAT3 and siFector were purchased from Dharmacon and B-Bridge, respectively. RNA interference (RNAi) screens were performed according to the manufacturers' protocols. Primers used for reverse transcription (RT)–PCR were as follows: mLIV1; 5′-AAAAATCCTAGAACTAGTCTAGGGAAAGGA-3′ (sense), 5′-CCTTCAGCTCCTCTCGAGAGTAGCGCTGGC-3′ (antisense), mSOCS3; 5′-ATGGTCACCCACAGCAAGTTT-3′ (sense), 5′-TTAAAGTGGAGCATCATACTG-3′ (antisense), mG3PDH; 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (sense), 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (antisense), hSTAT3; 5′-GATCTGAATGG AAACAACCAG-3′ (sense), 5′-GCACACTTTAATTTTAAGCTG-3′ (antisense), hLIV1; 5′-GCAATGGCGAGGAAGTTATCT-3′ (sense), 5′-CTATTGTCTCTAGAAAGTGAG-3′ (antisense), hSOCS3; 5′-ATGGTCACCCACAGCAAGTTT-3′ (sense), 5′-TTAAAGC GGGGCATCGTACTG-3′ (antisense), hCyclophilin-B; 5′-ATGCTGCGCCTCTCCG AACGC-3′ (sense), 5′-GTACTCCTTGGCGATGGCAAA-3′ (antisense), hG3PDH; 5′-TGAAGGTCGGAGTCAACGGAT-3′ (sense), 5′-CATGTGGGCCATGAGGTCCAC-3′ (antisense).

Cell-tracing experiments

Cell-tracing experiments were performed essentially as described previously[10]. 100 pl of 0.5% DMNB-caged fluorescein-dextran (molecular weight 10,000, Molecular Probes) was injected into the yolk cell of one-cell-stage embryos previously injected with 10 ng of the indicated morpholino. To uncage the dye, a beam of ultraviolet light ($\lambda < 360$ nm), generated using a DAPI filter set, was directed for 1 s at the dorsal or lateral blastoderm margin.

Transplantation experiments

Transplantation experiments were performed essentially as described previously[10]. Donor embryos were injected into the yolk cell of one-cell-stage embryos with 100 pl of 0.5% rhodamine-dextran (m_r 10,000, Molecular Probes) or with 100 pl of 0.5% fluorescein-dextran (m_r 10,000, Molecular Probes), and 10 ng of the indicated morpholino. A small population of deep cells (10–30 cells) from the embryonic shield of donor embryos was transplanted into the embryonic shield of host embryos at the same developmental stage.

Reporter assays

Reporter assays were performed essentially as described previously[14]. The reporter construct pGL3-E-cadh promoter (2.5 pg), which corresponds to the −178 to +92 human E-cadherin promoter fragment, was injected into one-cell-stage embryos with or without the synthetic RNA and/or the antisense morpholino. Firefly luciferase (Luc) and Renilla reniformis luciferase (Rlluc) activities were measured using the Dual Luciferase Reporter Assay System (Promega) at the shield stage (6 hours post fertilization, h.p.f.), according to the manufacturer's protocol. Luc activity was always normalized to the Rlluc activity.

1. Thiery, J. P. Epithelial-mesenchymal transitions in tumour progression. Nature Rev. Cancer 2, 442–454 (2002).
2. Savagner, P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 23, 912–923 (2001).
3. Darnell, J. E. Jr. STATs and gene regulation. Science 277, 1630–1635 (1997).
4. Bromberg, J. & Darnell, J. E. Jr The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19, 2468–2473 (2000).
5. Hirano, T., Ishihara, K. & Hibi, M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 19, 2548–2556 (2000).
6. Kamimura, D. & Hirano, T. in Signal Transducers and Activators of Transcription (STATs): Activation and Biology (eds Sehgal, P. B., Levy, D. E. & Hirano, T.) 155–175 (Kluwer Academic, Dordrecht, 2003).
7. Sano, S. et al. Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. EMBO J. 18, 4657–4668 (1999).
8. Silver, D. L. & Montell, D. J. Paracrine signaling through the JAK/STAT pathway activates invasive behavior of ovarian epithelial cells in Drosophila. Cell 107, 831–841 (2001).
9. Yamashita, S. & Hirano, T. in Signal Transducers and Activators of Transcription (STATs): Activation and Biology (eds Sehgal, P. B., Levy, D. E. & Hirano, T.) 595–607 (Kluwer Academic, Dordrecht, 2003).
10. Yamashita, S. et al. Stat3 controls cell movements during zebrafish gastrulation. Dev. Cell 2, 363–375 (2002).
11. Manning, D. L., Daly, R. J., Lord, P. G., Kelly, K. F. & Green, C. D. Effects of oestrogen on the expression of a 4.4 kb mRNA in the ZR-75-1 human breast cancer cell line. Mol. Cell. Endocrinol. 59, 205–212 (1988).
12. Manning, D. L. et al. Oestrogen-regulated genes in breast cancer: association of pLIV1 with lymph node involvement. Eur. J. Cancer 30A, 675–678 (1994).
13. Taylor, K. M., Morgan, H. E., Johnson, A., Hadley, L. J. & Nicholson, R. I. Structure-function analysis of LIV-1, the breast cancer-associated protein that belongs to a new subfamily of zinc transporters. Biochem. J. 375, 51–59 (2003).
14. Batlle, E. et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nature Cell Biol. 2, 84–89 (2000).
15. Cano, A. et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nature Cell Biol. 2, 76–83 (2000).
16. Taylor, K. M. & Nicholson, R. I. The LZT proteins; the LIV-1 subfamily of zinc transporters. Biochim. Biophys. Acta 1611, 16–30 (2003).
17. Fukada, T. et al. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. Immunity 5, 449–460 (1996).
18. Nasevicius, A. & Ekker, S. C. Effective targeted gene 'knockdown' in zebrafish. Nature Genet. 26, 216–220 (2000).
19. Kozlowski, D. J. & Weinberg, E. S. Photoactivatable (caged) fluorescein as a cell tracer for fate mapping in the zebrafish embryo. Methods Mol. Biol. 135, 349–355 (2000).
20. Dominguez, D. et al. Phosphorylation regulates the subcellular location and activity of the snail transcriptional repressor. Mol. Cell. Biol. 23, 5078–5089 (2003).
21. Van Doren, M. et al. Fear of intimacy encodes a novel transmembrane protein required for gonad morphogenesis in Drosophila. Development 130, 2355–2364 (2003).
22. Thisse, C., Thisse, B., Schilling, T. F. & Postlethwait, J. H. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 119, 1203–1215 (1993).
23. Thisse, C., Thisse, B. & Postlethwait, J. H. Expression of snail2, a second member of the zebrafish snail family, in cephalic mesendoderm and presumptive neural crest of wild-type and spadetail mutant embryos. Dev. Biol. 172, 86–99 (1995).
24. Solnica-Krezel, L., Stemple, D. L. & Driever, W. Transparent things: cell fates and cell movements during early embryogenesis of zebrafish. Bioessays 17, 931–939 (1995).
25. Blanco, M. J. et al. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. Oncogene 21, 3241–3246 (2002).
26. Bowman, T., Garcia, R., Turkson, J. & Jove, R. STATs in oncogenesis. Oncogene 19, 2474–2488 (2000).
27. Ciruna, B. & Rossant, J. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev. Cell 1, 37–49 (2001).
28. Peinado, H., Quintanilla, M. & Cano, A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. J. Biol. Chem. 278, 21113–21123 (2003).

Identification of the pollen determinant of S-RNase-mediated self-incompatibility

Paja Sijacic[1], Xi Wang[2]*, Andrea L. Skirpan[2], Yan Wang[3], Peter E. Dowd[2], Andrew G. McCubbin[2]*, Shihshieh Huang[4] & Teh-hui Kao[1,2,3]

[1]Integrative Biosciences Graduate Degree Program, [2]Department of Biochemistry and Molecular Biology, [3]Intercollege Graduate Program in Plant Physiology, The Pennsylvania State University, 403 Althouse Laboratory, University Park, Pennsylvania 16802, USA
[4]Mystic Research, Monsanto Company, 62 Maritime Drive, Mystic, Connecticut 06355, USA

* Present addresses: Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109, USA (X.W.); School of Biological Sciences and Center for Reproductive Biology, Washington State University, Pullman, Washington 99164, USA (A.G.M.)

Many flowering plants have adopted self-incompatibility mechanisms to prevent inbreeding and promote out-crosses[1]. In the Solanaceae, Rosaceae and Scrophulariaceae, two separate genes at the highly polymorphic *S*-locus control self-incompatibility interactions: the *S-RNase* gene encodes the pistil determinant and the previously unidentified *S*-gene encodes the pollen deter-

minant[2–4]. S-RNases interact with pollen S-allele products to inhibit the growth of self-pollen tubes in the style. Pollen-expressed F-box genes showing allelic sequence polymorphism have recently been identified near to the S-RNase gene in members of the Rosaceae and Scrophulariaceae[5–8]; but until now have not been directly shown to encode the pollen determinant. Here we report the identification and characterization of *PiSLF*, an S-locus F-box gene of *Petunia inflata* (Solanaceae). We show that transformation of S_1S_1, S_1S_2 and S_2S_3 plants with the S_2-allele of *PiSLF* causes breakdown of their pollen function in self-incompatibility. This breakdown of pollen function is consistent with 'competitive interaction', in which pollen carrying two different pollen S-alleles fails to function in self-incompatibility[1,9,10]. We conclude that *PiSLF* encodes the pollen self-incompatibility determinant.

To maintain self-incompatibility, genes encoding the pollen and pistil specificity determinants must be tightly linked at the S-locus. Otherwise, intergenic recombination would cause the breakdown of self-incompatibility by generating different pollen and pistil specificities within a single S-haplotype. We thus searched for the pollen S-gene of *P. inflata* on a previously constructed 328-kilobase (kb) bacterial artificial chromosome (BAC) contig that contains S_2-*RNase*[11,12]. Sequence analysis of this contig revealed an open reading frame for 389 amino acids, located ~161 kb downstream of the S_2-RNase gene. The predicted protein contains an F-box motif at its amino terminus, and was named $PiSLF_2$ (*P. inflata* S-locus F-box, with '2' indicating the allele number).

To ascertain whether *PiSLF* is expressed, an 888-base-pair (bp) fragment of the $PiSLF_2$ coding region (without the F-box coding sequence) was used as a probe in RNA gel blot analysis (Fig. 1a). A ~1.4-kb transcript was detected in anthers, mature pollen and pollen tubes, but not in any of the other tissues examined. During pollen development, the transcript was first detected in stage-three anthers (containing microspores undergoing mitosis), peaked in stage-four anthers (containing immature bicellular pollen) and subsequently declined. Although this expression pattern is somewhat unexpected for a gene that may function during pollen-tube growth in the style, transcript abundance may not reflect protein levels and the pollen tubes obtained by *in vitro* germination may not reflect the situation *in vivo*. Genomic DNA blot analysis revealed an S-haplotype-specific restriction-fragment length polymorphism (RFLP) (Fig. 1b).

We used reverse transcription polymerase chain reaction (RT–PCR) to isolate a ~1.3-kb complementary DNA fragment of $PiSLF_2$ from total RNA of S_2 pollen; the cDNA sequence is identical to the sequence of the corresponding region of $PiSLF_2$ in the 328-kb contig. To determine the degree of allelic sequence diversity of *PiSLF*, we used RT–PCR to obtain cDNAs for $PiSLF_1$ and $PiSLF_3$ from the total RNA isolated from S_1 and S_3 pollen, respectively. The cDNA obtained for each S-genotype was used as a probe in genomic DNA blot analysis, and generated the same RFLP pattern as shown in Fig. 1b (results not shown), confirming that these cDNAs correspond to alleles of *PiSLF*. The deduced amino acid sequences of the S_1-, S_2- and S_3-alleles of *PiSLF* (Supplementary Fig. S1) show 10.3% to 11.6% allelic sequence diversity, which is lower than that of the S-RNase (18.9% to 26.4%)[13], but higher than that of two other S-linked F-box proteins of *P. inflata* that we had previously identified (3.2% to 5.1% for A113 and 1.8% to 3.4% for A134)[14].

To ascertain whether *PiSLF* encodes the pollen S-determinant, we took advantage of a well-documented cause of breakdown of pollen S-function in the Solanaceae, termed competitive interaction. Competitive interaction occurs in self-incompatible plants that carry two different S-haplotypes when one of the S-loci, or some part of it that contains the pollen S-allele, is duplicated, or if these plants become tetraploids[1,9,10]. Among the pollen grains produced, those carrying two different pollen S-alleles (that is, heteroallelic pollen), but not those carrying two copies of the same pollen S-allele (that is, homoallelic pollen), fail to function in self-incompatibility. How different pollen S-alleles 'compete' to cause the breakdown of self-incompatibility function is not understood. We reasoned that if *PiSLF* is the pollen S-gene, then introducing its S_2-allele into self-incompatible plants of S_1S_1 genotype should render the transgenic plants self-compatible. As illustrated in Fig. 2a, if an S_1S_1 plant carries one copy of the $PiSLF_2$ transgene, half the pollen produced will carry the transgene. Upon self-pollination, pollen carrying the transgene will be compatible with the pistil because of competitive interaction between the endogenous S_1-allele and the introduced S_2-allele of *PiSLF*. In contrast, pollen not carrying the transgene will
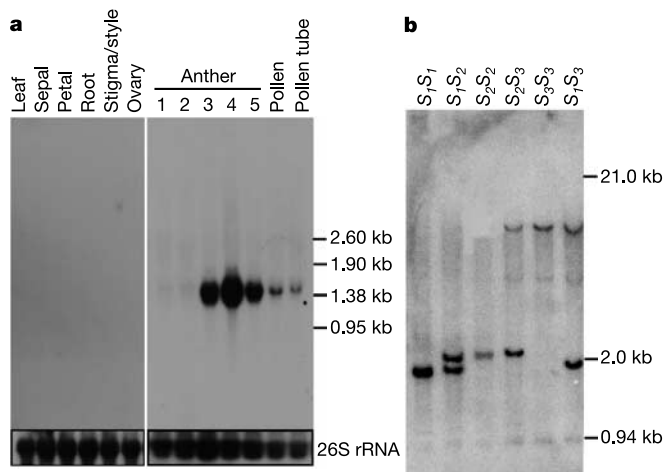

Figure 1 Characterization of *PiSLF*. **a**, RNA gel blot analysis of expression of *PiSLF*. Each lane contains 20 μg of total RNA. The anther stages are defined by flower-bud size as previously described[21]. **b**, Genomic DNA blot analysis of six S-genotypes. Each lane contains 15 μg of genomic DNA digested with *Eco*RI. The probe used for the upper blot in **a** and for the blot in **b** was an 888-bp fragment of $PiSLF_2$ without the F-box motif region. DNA and RNA size markers are indicated.
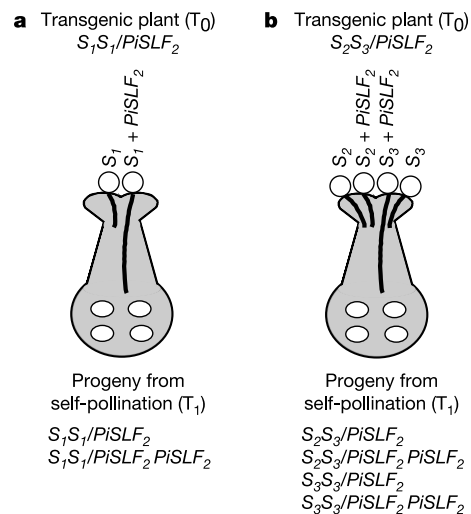

Figure 2 Schematic representation of transformation experiments to ascertain the function of *PiSLF*. **a**, Self-incompatibility behaviour of an S_1S_1 transgenic plant carrying a single copy of the $PiSLF_2$ transgene. **b**, Self-incompatibility behaviour of an S_2S_3 transgenic plant carrying a single copy of the $PiSLF_2$ transgene. The genotypes of pollen produced, the predicted S-genotypes of the progeny resulting from self-pollination, and inheritance of the transgene are indicated.

behave normally and be rejected by the pistil. Thus, all the progeny from self-pollination will carry the $PiSLF_2$ transgene and be self-compatible.

A ~4.3-kb fragment of $PiSLF_2$ (Supplementary Fig. S2) was introduced into S_1S_1 plants via *Agrobacterium*-mediated transformation. Genomic DNA blot analysis showed that of the 25 T_0 transgenic plants obtained, 20 (for example, the plant designated $S_1S_1/PiSLF_2$-3) carried two or more copies of the transgene, and five (designated $S_1S_1/PiSLF_2$-1, -2, -4, -5, -6) carried a single copy (Fig. 3a). The mature flowers of these five transgenic plants were self-pollinated and all pollinations resulted in large fruits, with an average seed number per fruit ranging from 115 to 170, comparable to those obtained from compatible pollination between wild-type plants. These five plants were further analysed to determine whether the breakdown of self-incompatibility was caused by competitive interaction.

First, we designed two pairs of PCR primers, one specific to $PiSLF_1$ and the other to $PiSLF_2$, and used RT–PCR to examine the expression of both alleles of $PiSLF$ in pollen. Products specific to $PiSLF_1$ and $PiSLF_2$ were obtained in all five plants (Fig. 3b), demonstrating that both endogenous $PiSLF_1$ and the $PiSLF_2$ transgene were expressed. This is consistent with the suggestion that competitive interaction is not due to silencing of pollen S-alleles present in heteroallelic pollen[15].

Next, we examined whether it was the pollen or the pistil that failed to function in self-incompatibility. When a wild-type S_1S_1

plant was pollinated by pollen from $S_1S_1/PiSLF_2$-1, large fruits were obtained with an average seed number per fruit of 120. However, when $S_1S_1/PiSLF_2$-1 was pollinated by pollen from the wild-type S_1S_1 plant, no fruits were obtained. Similar results were found when $S_1S_1/PiSLF_2$-2 was reciprocally crossed with the wild-type S_1S_1 plant. Thus, the presence and expression of the $PiSLF_2$ transgene caused the breakdown of the pollen function, but not of the pistil function, in self-incompatibility.

We raised 30 progeny (T_1 plants) from self-pollination of $S_1S_1/PiSLF_2$-1 and examined the inheritance of the $PiSLF_2$ transgene. PCR analysis revealed that all 30 plants carried the $PiSLF_2$ transgene (the results for eight T_1 plants are shown in Fig. 3c). The presence of the transgene in these eight T_1 plants was further confirmed by genomic blot analysis (Fig. 3d). The finding that all the progeny from self-pollination of $S_1S_1/PiSLF_2$-1 inherited the $PiSLF_2$ transgene is consistent with the breakdown of self-incompatibility resulting from competitive interaction between $PiSLF_1$ and $PiSLF_2$ (Fig. 2a). Moreover, all eight T_1 plants were self-compatible and set large fruits with an average seed number per fruit ranging from 132 to 165. We also pollinated a wild-type S_1S_1 plant with pollen from $S_1S_1/PiSLF_2$-1 and -2, and found from PCR analysis that all 50 T_1 plants of $S_1S_1/PiSLF_2$-1 and all 49 T_1 plants of $S_1S_1/PiSLF_2$-2 carried the transgene (results not shown).

To further examine whether the $PiSLF_2$ transgene specifically affects the self-incompatibility behaviour of heteroallelic pollen, we introduced the $PiSLF_2$ transgene into S_1S_2 and S_2S_3 plants (the predicted results for S_2S_3 are shown in Fig. 2b). Self-pollinations of three S_1S_2 transgenic plants and three S_2S_3 transgenic plants, all of which carried a single copy of the transgene, resulted in large fruits (with an average seed number per fruit ranging from 125 to 178). All of these plants rejected pollen from wild-type plants of the same S-genotype. The progeny from self-pollinations of $S_1S_2/PiSLF_2$-1 and $S_2S_3/PiSLF_2$-1 were analysed for the S-genotype and inheritance of the transgene. For $S_1S_2/PiSLF_2$-1, all 46 T_1 plants were either S_1S_1 (16) or S_1S_2 (30); for $S_2S_3/PiSLF_2$-1, all 48 T_1 plants were either S_3S_3 (18) or S_2S_3 (30). Figure 4 shows representative results of the progeny of $S_2S_3/PiSLF_2$-1. The absence of S_2S_2 genotype in the progeny of these two transgenic plants suggests that S_2 pollen was rejected, and hence the $PiSLF_2$ transgene does not affect the self-incompatibility function of S_2 pollen. Moreover, all of the 94 T_1 plants examined carried the $PiSLF_2$ transgene, suggesting that only S_1 pollen carrying the transgene and S_3 pollen carrying the transgene were compatible with S_1S_2 and S_2S_3 pistils, respectively.

From the results of the analyses of the T_0 and T_1 transgenic plants, we conclude that $PiSLF$ encodes the pollen determinant of S-RNase-mediated self-incompatibility. The finding that an F-box protein
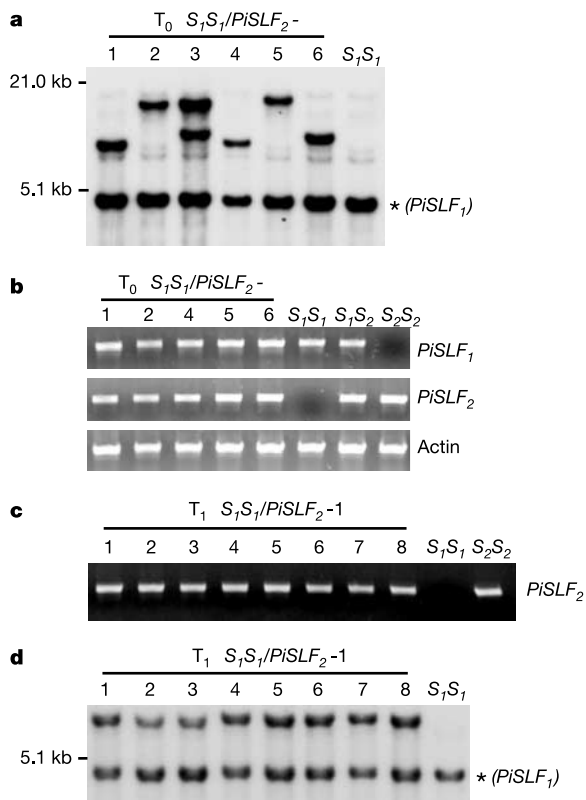

Figure 3 Analyses of S_1S_1 transgenic plants carrying $PiSLF_2$ transgene. **a**, Transgene copy number of six T_0 plants. Genomic DNA was digested with *Xba*I and hybridized with the same $PiSLF_2$ probe as that used in Fig. 1. **b**, RT–PCR analysis of endogenous $PiSLF_1$ and the $PiSLF_2$ transgene in the five T_0 plants ($S_1S_1/PiSLF_2$-1, -2, -4, -5, -6) carrying a single copy of the transgene. **c**, PCR analysis of inheritance of the $PiSLF_2$ transgene in eight T_1 plants from self-pollination of $S_1S_1/PiSLF_2$-1. **d**, DNA blot analysis to confirm the results shown in **c**. In **a** and **d**, asterisks denote the endogenous $PiSLF_1$ gene, and additional hybridizing fragment(s) correspond to the $PiSLF_2$ transgene. Wild-type plants are included as controls.
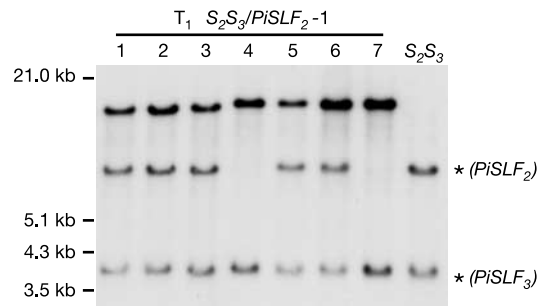

Figure 4 Analysis of progeny of an S_2S_3 transgenic plant, $S_2S_3/PiSLF_2$-1, carrying a single copy of the $PiSLF_2$ transgene. Genomic DNA was digested with *Xba*I and hybridized with the same $PiSLF_2$ probe used in Fig. 1. Asterisks denote the endogenous $PiSLF_2$ and $PiSLF_3$ genes indicated in parentheses. The additional hybridizing fragment in each lane corresponds to the $PiSLF_2$ transgene. A wild-type plant of S_2S_3 genotype is included as a control.

determines pollen specificity in self-incompatibility interactions has important implications for how S-RNases specifically inhibit the growth of self-pollen tubes. It has been shown that RNase activity is required for the function of S-RNases[16], and that both self and non-self S-RNases are taken up by pollen tubes growing in a pistil[17]. Therefore, it seems that only self S-RNase is able to function inside a pollen tube, degrading RNA substrate(s) to inhibit pollen-tube growth. This raises the question as to how the toxicity of non-self S-RNases is neutralized.

Recently, it has been shown that an S-locus F-box protein of *Antirrhinum* (Scrophulariaceae), AhSLF-S$_2$, is a likely component of the SCF complex (composed of Skp1, Cullin1, Rbx1 and an F-box protein)[18], which is involved in ubiquitin-mediated protein degradation by the 26S proteasome[19]. Interestingly, AhSLF-S$_2$ interacts with both self and non-self S-RNases, but appears to mediate degradation of only non-self S-RNases[18]. Because there are multiple S-linked F-box genes in *Antirrhinum*[8] and *P. inflata*[14], whether *AhSLF* is an orthologue of *PiSLF* remains to be determined. If PiSLF is a component of the SCF complex, its interaction with a domain common to all S-RNases might lead to ubiquitination and degradation, but this interaction would be prevented by an alternate specific interaction between PiSLF and its cognate self S-RNase. □

Methods

DNA and RNA gel blot analyses

Isolation of genomic DNA from young leaves and genomic DNA blot analysis were carried out as previously described[14], as was isolation of total RNA and RNA gel blot analysis[20].

Construction of transgene and *Agrobacterium*-mediated transformation

An ~11-kb DNA fragment containing *PiSLF$_2$* was released from BAC clone 145J16 (ref. 12) by *Bam*HI digestion and cloned into the *Bam*HI site of pBluescript KS + (Stratagene) to yield pBS-11K. pBS-11K was digested with *Sal*I, made blunt-ended by Klenow enzyme, and digested with *Xba*I to release a ~4.3-kb fragment containing *PiSLF$_2$*. A Ti plasmid, pBI101 (Clontech), was digested with *Sac*I, made blunt-ended by T4 DNA polymerase, and digested with *Xba*I. The ~4.3-kb DNA fragment containing *PiSLF$_2$* was ligated into the *Xba*I site and the blunt-end site of pBI101 (see Supplementary Fig. S2). The recombinant Ti plasmid was electroporated into *Agrobacterium tumefaciens* strain LBA4404 using a Cell-Porator (Life Technologies). The transformation of *P. inflata* leaf strips with *Agrobacterium* was carried out as described previously[2].

PCR and RT–PCR

To clone *PiSLF$_1$*, *PiSLF$_2$* and *PiSLF$_3$* by RT–PCR, cDNA was separately synthesized from 5 μg of total RNA isolated from S$_1$, S$_2$ and S$_3$ pollen using 200 units of SuperScript II RNaseH-reverse transcriptase (Invitrogen). PCR was carried out on the cDNA of each S-genotype in a standard reaction buffer containing 0.25 μM each of forward primer (5′-GATCCAGTTGAATGAGATGG-3′) and reverse primer, oligo (dT)$_{17}$, and two units of Taq DNA polymerase (Fisher Scientific). The reaction mixture was denatured at 94 °C for 1 min, then subjected to 30 cycles, with each cycle consisting of denaturation at 94 °C for 45 s, annealing at 45 °C for 45 s and extension at 72 °C for 1 min. After the last cycle, the sample was kept at 72 °C for an additional 10 min. PCR products were gel-purified and ligated into pGEM-T Easy vector (Promega).

To assess the presence of the *PiSLF$_2$* transgene in T$_0$ and T$_1$ transgenic plants, PCR was carried out on 5 ng of genomic DNA isolated from each plant as described above, except that the forward primer was 5′-TATTAACATTTCCAGTGAAATCTCTTAT-3′, the reverse primer was 5′-GTATCAGGCATTTTCATATCATGAAAC-3′ and the annealing temperature was 58 °C. To examine the expression of *PiSLF$_1$* and *PiSLF$_2$* in T$_0$ transgenic plants, cDNA was synthesized as described above. PCR conditions were the same as those described above except that for *PiSLF$_1$*, the annealing temperature was 63 °C and the primers used were: 5′-TGTTAACATTTCCCGTTAAATCTCTCCT-3′ (forward primer) and 5′-ATCTTTTACTGGATCAATAGAGCTTATTGG-3′ (reverse primer). To use actin as the control in RT–PCR, two primers, 5′-GGCATCACACTTTCTACAATGAGC-3′ (forward) and 5′-GATATCCACATCACATTTCATGAT-3′ (reverse), were designed on the basis of the sequence of pollen-expressed *Arabidopsis* actin 12 (At3g46520).

1. de Nettancourt, D. *Incompatibility and Incongruity in Wild and Cultivated Plants* (Springer, Berlin, 2001).
2. Lee, H.-S., Huang, S. & Kao, T.-h. S proteins control rejection of incompatible pollen in *Petunia inflata*. *Nature* **367**, 560–563 (1994).
3. Murfett, J., Atherton, T. L., Mou, B., Gasser, C. S. & McClure, B. A. S-RNase expressed in transgenic *Nicotiana* causes S-allele-specific pollen rejection. *Nature* **367**, 563–566 (1994).
4. Kao, T.-h. & Tsukamoto, T. The molecular and genetic bases of S-RNase-based self-incompatibility. *Plant Cell* advance online publication 9 March 2004 (doi:10.1105/tpc.016154).
5. Lai, Z. *et al.* 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 (2002).
6. Entani, T. *et al.* 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 (2003).
7. Ushijima, K. *et al.* 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 (2003).
8. Zhou, J. *et al.* Structural and transcriptional analysis of S-locus F-box (*SLF*) genes in *Antirrhinum*. *Sex. Plant Reprod.* **16**, 165–177 (2003).
9. Golz, J. F., Su, V., Clarke, A. E. & Newbigin, E. A molecular description of mutations affecting the pollen component of the *Nicotiana alata S* locus. *Genetics* **152**, 1123–1135 (1999).
10. Golz, J. F., Oh, H.-Y., Su, V., Kusaba, M. & Newbigin, E. 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 (2001).
11. McCubbin, A. G., Zuniga, C. & Kao, T.-h. Construction of a binary artificial chromosome library of *Petunia inflata* and the identification of large genomic fragments linked to the self-incompatibility (S-) locus. *Genome* **43**, 820–826 (2000).
12. Wang, Y. *et al.* Chromosome walking in the *Petunia inflata* self-incompatibility (S-) locus and gene identification in an 881-kb contig containing S$_2$-RNase. *Plant Mol. Biol* (in the press).
13. Ai, Y. *et al.* Self-incompatibility in *Petunia inflata*: isolation and characterization of cDNAs encoding three S-allele-associated proteins. *Sex. Plant Reprod.* **3**, 130–138 (1990).
14. Wang, Y., Wang, X., McCubbin, A. G. & Kao, T.-h. Genetic mapping and molecular characterization of the self-incompatibility (S-) locus in *Petunia inflata*. *Plant Mol. Biol.* **53**, 565–580 (2003).
15. Luu, D.-T. *et al.* Rejection of S-heteroallelic pollen by a dual-specific S-RNase in *Solanum chacoense* predicts a multimeric self-incompatibility pollen component. *Genetics* **159**, 329–335 (2001).
16. Huang, S., Lee, H.-S., Karunanandaa, B. & Kao, T.-h. Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. *Plant Cell* **6**, 1021–1028 (1994).
17. Luu, D.-T., Qin, K., Morse, D. & Cappadocia, M. S-RNase uptake by compatible pollen tubes in gametophytic self-incompatibility. *Nature* **407**, 649–651 (2000).
18. Qiao, H. *et al.* 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 (2004).
19. Bai, C. *et al.* Skp1 connects cell cycle regulation to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86**, 263–274 (1996).
20. Skirpan, A. L. *et al.* Isolation and characterization of kinase interacting protein 1, a pollen protein that interacts with the kinase domain of PRK1, a receptor-like kinase of petunia. *Plant Physiol.* **126**, 1480–1492 (2001).
21. Mu, J.-H., Lee, H.-S. & Kao, T.-h Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. *Plant Cell* **6**, 709–721 (1994).

••

Self-incompatibility triggers programmed cell death in *Papaver* pollen

Steven G. Thomas & Veronica E. Franklin-Tong

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

....

Sexual reproduction in many angiosperm plants involves self-incompatibility (SI), which is one of the most important mechanisms to prevent inbreeding. SI is genetically controlled by the S-locus, and involves highly specific interactions during pollination between pollen and the pistil on which it lands. This results in the rejection of incompatible ('self') pollen, whereas compatible ('non-self') pollen is allowed to fertilize the plant[1]. In *Papaver rhoeas*, S-proteins encoded by the stigma component of the S-locus interact with incompatible pollen, triggering a Ca^{2+}-dependent signalling network[2–7], resulting in the inhibition of pollen-tube growth. Programmed cell death (PCD) is a