

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)<sup>10</sup>, and pCS2 + mouse Snail (*NotI*, SP6)<sup>14</sup>. The cDNA sequence of full-length mouse Snail (1–264)<sup>20</sup> and C-terminus-deleted mouse Snail (1–151)<sup>20</sup> 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<sup>17</sup>. Cells were starved with factor-free medium for 6 h followed by stimulation with medium containing 100 ng ml<sup>-1</sup> 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 siFactor 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'-CCTTCAGCTCTCTCGAGAGTAGCGCTGGC-3' (antisense), mSOCS3; 5'-ATGGTCACCCACAGCAAGTTT-3' (sense), 5'-TTAAAGTGGAGCATCATACTG-3' (antisense), mG3PDH; 5'-TGAAGTCCGGTGTGAACGGATTGGC-3' (sense), 5'-CATGTAGGCCATGAGGTCCACCAC-3' (antisense), hSTAT3; 5'-GATCTGAATGG AAACAACCAAG-3' (sense), 5'-GCACACTTAAATTTAAAGCTG-3' (antisense), hLIV1; 5'-GCAATGGCGAGGAAGTTATCT-3' (sense), 5'-CTATTGTCTCTAGAAAGTGAAG-3' (antisense), hSOCS3; 5'-ATGGTCACCCACAGCAAGTTT-3' (sense), 5'-TTAAAGC GGGGCATCGTACTG-3' (antisense), hCyclophilin-B; 5'-ATGCTGGCCCTCTCCG AACGC-3' (sense), 5'-GTACTCCTTGGCGATGGCAAA-3' (antisense), hG3PDH; 5'-TGAAGTCCGGAGTCAACGGAT-3' (sense), 5'-CATGTGGCCATGAGTCCAC-3' (antisense).

## Cell-tracing experiments

Cell-tracing experiments were performed essentially as described previously<sup>10</sup>. 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<sup>10</sup>. 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<sup>14</sup>. 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 (Rluc) 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 Rluc activity.

Received 19 February; accepted 5 April 2004; doi:10.1038/nature02545.  
Published online 5 May 2004.

- Thiery, J. P. Epithelial-mesenchymal transitions in tumour progression. *Nature Rev. Cancer* **2**, 442–454 (2002).
- Savagner, P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays* **23**, 912–923 (2001).
- Darnell, J. E. Jr. STATs and gene regulation. *Science* **277**, 1630–1635 (1997).
- Bromberg, J. & Darnell, J. E. Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* **19**, 2468–2473 (2000).
- 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).
- 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).
- 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).
- 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).
- 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).
- Yamashita, S. *et al.* Stat3 controls cell movements during zebrafish gastrulation. *Dev. Cell* **2**, 363–375 (2002).
- 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).
- 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).
- 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).
- Batle, 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).
- Cano, A. *et al.* The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature Cell Biol.* **2**, 76–83 (2000).
- Taylor, K. M. & Nicholson, R. I. The LZT proteins; the LIV-1 subfamily of zinc transporters. *Biochim. Biophys. Acta* **1611**, 16–30 (2003).
- 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).
- Nasevicius, A. & Ekker, S. C. Effective targeted gene 'knockdown' in zebrafish. *Nature Genet.* **26**, 216–220 (2000).
- 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).
- Dominguez, D. *et al.* Phosphorylation regulates the subcellular location and activity of the snail transcriptional repressor. *Mol. Cell. Biol.* **23**, 5078–5089 (2003).
- Van Doren, M. *et al.* Fear of intimacy encodes a novel transmembrane protein required for gonad morphogenesis in *Drosophila*. *Development* **130**, 2355–2364 (2003).
- 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).
- Thisse, C., Thisse, B. & Postlethwait, J. H. Expression of snail2, a second member of the zebrafish snail family, in cephalic mesoderm and presumptive neural crest of wild-type and spadetail mutant embryos. *Dev. Biol.* **172**, 86–99 (1995).
- 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).
- Blanco, M. J. *et al.* Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* **21**, 3241–3246 (2002).
- Bowman, T., Garcia, R., Turkson, J. & Jove, R. STATs in oncogenesis. *Oncogene* **19**, 2474–2488 (2000).
- Ciruna, B. & Rossant, J. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev. Cell* **1**, 37–49 (2001).
- 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).

Supplementary Information accompanies the paper on [www.nature.com/nature](http://www.nature.com/nature).

**Acknowledgements** We thank A. G. De Herreros for the pGL3-E-cadh promoter plasmid and pDNA3-mm snail-HA plasmid, and many colleagues for providing reagents. We also thank R. Masuda and A. Kubota for secretarial assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

**Competing interests statement** The authors declare that they have no competing financial interests.

**Correspondence** and requests for materials should be addressed to T.H. (hirano@molonc.med.osaka-u.ac.jp). The zebrafish *LIV1* cDNA sequence has been deposited in the GenBank database under accession number AB126260.

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

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

<sup>1</sup>Integrative Biosciences Graduate Degree Program, <sup>2</sup>Department of Biochemistry and Molecular Biology, <sup>3</sup>Intercollege Graduate Program in Plant Physiology, The Pennsylvania State University, 403 Althouse Laboratory, University Park, Pennsylvania 16802, USA

<sup>4</sup>Mystic Research, Monsanto Company, 62 Maritime Drive, Mystic, Connecticut 06355, USA

\* Present address: 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<sup>1</sup>. 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<sup>2-4</sup>. 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<sup>5-8</sup>; 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*<sub>1</sub>*S*<sub>1</sub>, *S*<sub>1</sub>*S*<sub>2</sub> and *S*<sub>2</sub>*S*<sub>3</sub> plants with the *S*<sub>2</sub>-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<sup>1,9,10</sup>. We conclude that *PiSLF* encodes the pollen self-incompatibility determinant.

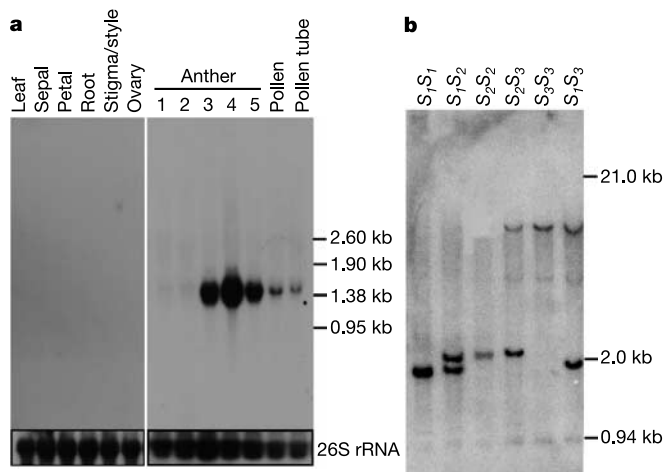
To maintain self-incompatibility, genes encoding the pollen and pistil specificity determinants must be tightly linked at the S-locus. Otherwise, intergenic recombination would cause the breakdown of self-incompatibility by generating different pollen and pistil specificities within a single S-haplotype. 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*<sub>2</sub>-*RNase*<sup>11,12</sup>. Sequence analysis of this contig revealed an open reading frame for 389 amino acids, located ~161 kb downstream of the *S*<sub>2</sub>-RNase gene. The predicted protein contains an F-box motif at its amino terminus, and was named *PiSLF*<sub>2</sub> (*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*<sub>2</sub> 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

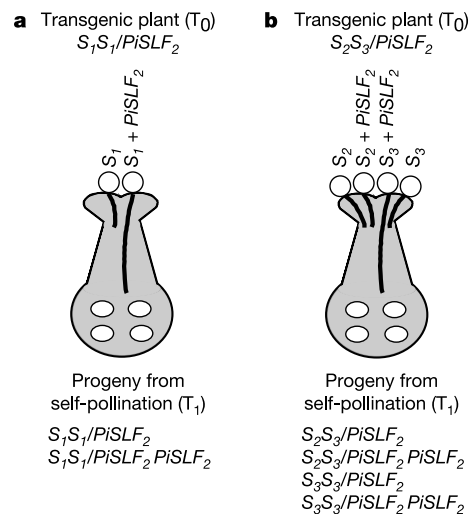
S-haplotype-specific restriction-fragment length polymorphism (RFLP) (Fig. 1b).

We used reverse transcription polymerase chain reaction (RT-PCR) to isolate a ~1.3-kb complementary DNA fragment of *PiSLF*<sub>2</sub> from total RNA of *S*<sub>2</sub> pollen; the cDNA sequence is identical to the sequence of the corresponding region of *PiSLF*<sub>2</sub> in the 328-kb contig. To determine the degree of allelic sequence diversity of *PiSLF*, we used RT-PCR to obtain cDNAs for *PiSLF*<sub>1</sub> and *PiSLF*<sub>3</sub> from the total RNA isolated from *S*<sub>1</sub> and *S*<sub>3</sub> 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*<sub>1</sub>-, *S*<sub>2</sub>- and *S*<sub>3</sub>-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%)<sup>13</sup>, 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)<sup>14</sup>.

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<sup>1,9,10</sup>. 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*<sub>2</sub>-allele into self-incompatible plants of *S*<sub>1</sub>*S*<sub>1</sub> genotype should render the transgenic plants self-compatible. As illustrated in Fig. 2a, if an *S*<sub>1</sub>*S*<sub>1</sub> plant carries one copy of the *PiSLF*<sub>2</sub> 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*<sub>1</sub>-allele and the introduced *S*<sub>2</sub>-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<sup>21</sup>. **b**, Genomic DNA blot analysis of six S-genotypes. Each lane contains 15 μg of genomic DNA digested with *EcoRI*. The probe used for the upper blot in **a** and for the blot in **b** was an 888-bp fragment of *PiSLF*<sub>2</sub> 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*<sub>1</sub>*S*<sub>1</sub> transgenic plant carrying a single copy of the *PiSLF*<sub>2</sub> transgene. **b**, Self-incompatibility behaviour of an *S*<sub>2</sub>*S*<sub>3</sub> transgenic plant carrying a single copy of the *PiSLF*<sub>2</sub> 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<sub>2</sub>* transgene and be self-compatible.

A ~4.3-kb fragment of *PiSLF<sub>2</sub>* (Supplementary Fig. S2) was introduced into *S<sub>1</sub>S<sub>1</sub>* plants via *Agrobacterium*-mediated transformation. Genomic DNA blot analysis showed that of the 25 *T<sub>0</sub>* transgenic plants obtained, 20 (for example, the plant designated *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-3*) carried two or more copies of the transgene, and five (designated *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-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<sub>1</sub>* and the other to *PiSLF<sub>2</sub>*, and used RT-PCR to examine the expression of both alleles of *PiSLF* in pollen. Products specific to *PiSLF<sub>1</sub>* and *PiSLF<sub>2</sub>* were obtained in all five plants (Fig. 3b), demonstrating that both endogenous *PiSLF<sub>1</sub>* and the *PiSLF<sub>2</sub>* 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<sup>15</sup>.

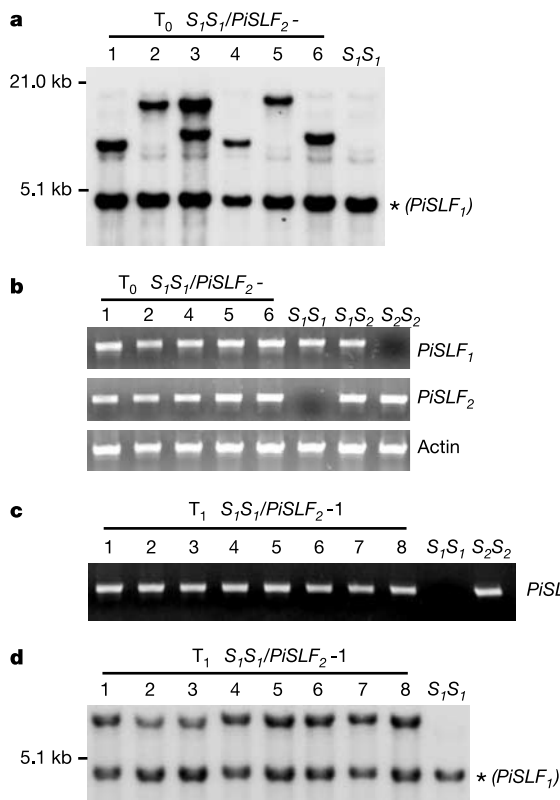
Next, we examined whether it was the pollen or the pistil that failed to function in self-incompatibility. When a wild-type *S<sub>1</sub>S<sub>1</sub>*

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

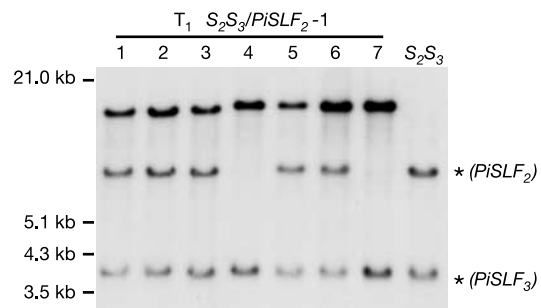
We raised 30 progeny (*T<sub>1</sub>* plants) from self-pollination of *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-1* and examined the inheritance of the *PiSLF<sub>2</sub>* transgene. PCR analysis revealed that all 30 plants carried the *PiSLF<sub>2</sub>* transgene (the results for eight *T<sub>1</sub>* plants are shown in Fig. 3c). The presence of the transgene in these eight *T<sub>1</sub>* plants was further confirmed by genomic blot analysis (Fig. 3d). The finding that all the progeny from self-pollination of *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-1* inherited the *PiSLF<sub>2</sub>* transgene is consistent with the breakdown of self-incompatibility resulting from competitive interaction between *PiSLF<sub>1</sub>* and *PiSLF<sub>2</sub>* (Fig. 2a). Moreover, all eight *T<sub>1</sub>* 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<sub>1</sub>S<sub>1</sub>* plant with pollen from *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-1* and -2, and found from PCR analysis that all 50 *T<sub>1</sub>* plants of *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-1* and all 49 *T<sub>1</sub>* plants of *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-2* carried the transgene (results not shown).

To further examine whether the *PiSLF<sub>2</sub>* transgene specifically affects the self-incompatibility behaviour of heteroallelic pollen, we introduced the *PiSLF<sub>2</sub>* transgene into *S<sub>1</sub>S<sub>2</sub>* and *S<sub>2</sub>S<sub>3</sub>* plants (the predicted results for *S<sub>2</sub>S<sub>3</sub>* are shown in Fig. 2b). Self-pollinations of three *S<sub>1</sub>S<sub>2</sub>* transgenic plants and three *S<sub>2</sub>S<sub>3</sub>* 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<sub>1</sub>S<sub>2</sub>/PiSLF<sub>2</sub>-1* and *S<sub>2</sub>S<sub>3</sub>/PiSLF<sub>2</sub>-1* were analysed for the *S*-genotype and inheritance of the transgene. For *S<sub>1</sub>S<sub>2</sub>/PiSLF<sub>2</sub>-1*, all 46 *T<sub>1</sub>* plants were either *S<sub>1</sub>S<sub>1</sub>* (16) or *S<sub>1</sub>S<sub>2</sub>* (30); for *S<sub>2</sub>S<sub>3</sub>/PiSLF<sub>2</sub>-1*, all 48 *T<sub>1</sub>* plants were either *S<sub>3</sub>S<sub>3</sub>* (18) or *S<sub>2</sub>S<sub>3</sub>* (30). Figure 4 shows representative results of the progeny of *S<sub>2</sub>S<sub>3</sub>/PiSLF<sub>2</sub>-1*. The absence of *S<sub>2</sub>S<sub>2</sub>* genotype in the progeny of these two transgenic plants suggests that *S<sub>2</sub>* pollen was rejected, and hence the *PiSLF<sub>2</sub>* transgene does not affect the self-incompatibility function of *S<sub>2</sub>* pollen. Moreover, all of the 94 *T<sub>1</sub>* plants examined carried the *PiSLF<sub>2</sub>* transgene, suggesting that only *S<sub>1</sub>* pollen carrying the transgene and *S<sub>3</sub>* pollen carrying the transgene were compatible with *S<sub>1</sub>S<sub>2</sub>* and *S<sub>2</sub>S<sub>3</sub>* pistils, respectively.

From the results of the analyses of the *T<sub>0</sub>* and *T<sub>1</sub>* 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<sub>1</sub>S<sub>1</sub>* transgenic plants carrying *PiSLF<sub>2</sub>* transgene. **a**, Transgene copy number of six *T<sub>0</sub>* plants. Genomic DNA was digested with *Xba*I and hybridized with the same *PiSLF<sub>2</sub>* probe as that used in Fig. 1. **b**, RT-PCR analysis of endogenous *PiSLF<sub>1</sub>* and the *PiSLF<sub>2</sub>* transgene in the five *T<sub>0</sub>* plants (*S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-1, -2, -4, -5, -6*) carrying a single copy of the transgene. **c**, PCR analysis of inheritance of the *PiSLF<sub>2</sub>* transgene in eight *T<sub>1</sub>* plants from self-pollination of *S<sub>1</sub>S<sub>1</sub>/PiSLF<sub>2</sub>-1*. **d**, DNA blot analysis to confirm the results shown in **c**. In **a** and **d**, asterisks denote the endogenous *PiSLF<sub>1</sub>* gene, and additional hybridizing fragment(s) correspond to the *PiSLF<sub>2</sub>* transgene. Wild-type plants are included as controls.



**Figure 4** Analysis of progeny of an *S<sub>2</sub>S<sub>3</sub>* transgenic plant, *S<sub>2</sub>S<sub>3</sub>/PiSLF<sub>2</sub>-1*, carrying a single copy of the *PiSLF<sub>2</sub>* transgene. Genomic DNA was digested with *Xba*I and hybridized with the same *PiSLF<sub>2</sub>* probe used in Fig. 1. Asterisks denote the endogenous *PiSLF<sub>2</sub>* and *PiSLF<sub>3</sub>* genes indicated in parentheses. The additional hybridizing fragment in each lane corresponds to the *PiSLF<sub>2</sub>* transgene. A wild-type plant of *S<sub>2</sub>S<sub>3</sub>* 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<sup>16</sup>, and that both self and non-self S-RNases are taken up by pollen tubes growing in a pistil<sup>17</sup>. 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<sub>2</sub>, is a likely component of the SCF complex (composed of Skp1, Cullin1, Rbx1 and an F-box protein)<sup>18</sup>, which is involved in ubiquitin-mediated protein degradation by the 26S proteasome<sup>19</sup>. Interestingly, AhSLF-S<sub>2</sub> interacts with both self and non-self S-RNases, but appears to mediate degradation of only non-self S-RNases<sup>18</sup>. Because there are multiple S-linked F-box genes in *Antirrhinum*<sup>8</sup> and *P. inflata*<sup>14</sup>, 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<sup>14</sup>, as was isolation of total RNA and RNA gel blot analysis<sup>20</sup>.

**Construction of transgene and *Agrobacterium*-mediated transformation**

An ~11-kb DNA fragment containing PiSLF<sub>2</sub> was released from BAC clone 145J16 (ref. 12) by BamHI digestion and cloned into the BamHI site of pBluescript KS + (Stratagene) to yield pBS-11K. pBS-11K was digested with SalI, made blunt-ended by Klenow enzyme, and digested with XbaI to release a ~4.3-kb fragment containing PiSLF<sub>2</sub>. A Ti plasmid, pBI101 (Clontech), was digested with SacI, made blunt-ended by T4 DNA polymerase, and digested with XbaI. The ~4.3-kb DNA fragment containing PiSLF<sub>2</sub> was ligated into the XbaI 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<sup>2</sup>.

**PCR and RT-PCR**

To clone PiSLF<sub>1</sub>, PiSLF<sub>2</sub> and PiSLF<sub>3</sub> by RT-PCR, cDNA was separately synthesized from 5 µg of total RNA isolated from S<sub>1</sub>, S<sub>2</sub> and S<sub>3</sub> pollen using 200 units of SuperScript II RNaseH<sup>-</sup> 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)<sub>17</sub>, 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<sub>2</sub> transgene in T<sub>0</sub> and T<sub>1</sub> 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'-TATTAACATTTCCAGTGAAATCTCTAT-3', the reverse primer was 5'-GTATCAGGCATTTTCATATCATGAAAC-3' and the annealing temperature was 58 °C. To examine the expression of PiSLF<sub>1</sub> and PiSLF<sub>2</sub> in T<sub>0</sub> transgenic plants, cDNA was synthesized as described above. PCR conditions were the same as those described above except that for PiSLF<sub>1</sub>, the annealing temperature was 63 °C and the primers used were: 5'-TGTTAATTTCCCGTTAAATCTCTCT-3' (forward primer) and 5'-ATCTTTTACTGGATCAATAGAGCTTATTGG-3' (reverse primer). To use actin as the control in RT-PCR, two primers, 5'-GGCATCACACTTCTACAATGAGC-3' (forward) and 5'-GATATCCACATCACATTTTCATGAT-3' (reverse), were designed on the basis of the sequence of pollen-expressed *Arabidopsis* actin 12 (At3g46520).

Received 17 December 2003; accepted 30 March 2004; doi:10.1038/nature02523.

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. Murrett, 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 glauca* 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<sub>2</sub>-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<sub>2</sub> 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).

Supplementary Information accompanies the paper on [www.nature.com/nature](http://www.nature.com/nature).

**Acknowledgements** We thank A. H. Omeis for the greenhouse work, J. Wang for routine laboratory assistance and the Monsanto Sequencing Facility for sequencing the BAC clones. This work was supported by a Predoctoral Fellowship for Students with Disabilities from the National Institutes of Health (P.E.D.) and by grants from the National Science Foundation (T.-h.K.).

**Competing interests statement** The authors declare that they have no competing financial interests.

**Correspondence** and requests for materials should be addressed to T.-h.K. (txk3@psu.edu).

**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<sup>1</sup>. In *Papaver rhoeas*, S-proteins encoded by the stigma component of the S-locus interact with incompatible pollen, triggering a Ca<sup>2+</sup>-dependent signalling network<sup>2–7</sup>, resulting in the inhibition of pollen-tube growth. Programmed cell death (PCD) is a