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Antisense phenotypes reveal a role for SHY, a pollen-specific leucine-rich repeat protein, in pollen tube growth

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Summary

***SHY*, a pollen-specific gene identified in a screen for genes upregulated at pollen germination (Guyon *et al.*, 2000), encodes a leucine-rich repeat (LRR) protein that is predicted to be secreted. To test if SHY plays an important role during pollen germination, we generated transgenic plants expressing an antisense (AS) copy of the *SHY* cDNA in pollen. Primary transformants exhibited poor seed set, but homozygous lines could be identified. In these lines, nearly all pollen tubes failed to reach the ovules; tube growth was arrested at the apex of the ovary and the pollen tubes exhibited abnormal callose deposits throughout the tube and in the tips. We show that a SHY::eGFP fusion protein is targeted to the cell wall. The structure of the SHY protein is nearly identical to other extracellular matrix glycoproteins that are composed of LRRs, such as the polygalacturonase inhibitor proteins (PGIP) of plants. PGIPs may function as defense proteins by inhibiting fungal endo-polygalacturonases, but enzyme assays with extracts of AS-*SHY* pollen do not support such an inhibitor role for SHY. The tomato ortholog of SHY interacts with a tomato receptor kinase (LePRK2) in yeast two-hybrid and pull-down assays (Tang *et al.*, 2002, 2004); this, and the AS-*SHY* phenotypes, suggest instead that SHY might function in a signal transduction pathway mediating pollen tube growth.**

Keywords: pollen germination, leucine-rich repeat, antisense.

Introduction

Pollen is released from the anther in a dehydrated state and upon contact with a compatible stigma it must adhere, hydrate, germinate, and elongate a pollen tube through the transmitting tract of the style in order to deposit the sperm cells in the embryo sac for fertilization (Taylor and Hepler, 1997). All of these processes involve accurate communication between the pollen and pistil cells, most of which take place within the extracellular matrix (ECM).

Although it is generally agreed that some or all of the mRNAs required for pollen tube growth are present in the mature grain (reviewed in Taylor and Hepler, 1997), several studies have shown that some mRNA synthesis occurs during the early moments of pollen germination (reviewed in Mascarenhas, 1993). In an effort to identify such early genes, we exploited the flavonol requirement for pollen germination in conditionally male fertile (CMF) petunia to isolate a set of petunia germinating pollen (PGP) cDNAs (Guyon *et al.*, 2000). Pollen from CMF plants lack flavonols but can be induced to germinate within 5 min of the addition of exogenous flavonols to an *in vitro* suspension of pollen, or to the stigma at pollination (Mo *et al.*, 1992; Vogt *et al.*, 1994). Expression of one of these PGP cDNAs, *S/D4*, was first detected following pollen mitosis I and its expression increased eightfold during the first 0.5 h of flavonol-induced germination. *S/D4* was not expressed in other floral tissues, or in vegetative tissues. *S/D4* has been renamed *SHY* to

reflect the pollen phenotype of the antisense (AS) plants reported here.

SHY encodes a 38.4-kDa protein with a 21 amino acid hydrophobic N-terminal sequence and 10 leucine-rich repeats (LRR) in tandem. The LRR is an evolutionarily conserved molecular recognition motif. A typical extracytoplasmic LRR (eLRR) comprises 24 residues with a periodic distribution of leucines interspersed among non-conserved, hydrophilic residues (Hocking *et al.*, 1998; Leckie *et al.*, 1999). Many LRR proteins are mosaics that can include one or more features: a membrane anchor, leucine zipper, nuclear binding site or kinase domain (Hocking *et al.*, 1998; Kobe and Deisenhofer, 1995). Adhesive proteins constitute the largest group in the LRR superfamily (Kobe and Deisenhofer, 1995) and include the small leucine-rich proteoglycans (SLRPs) that are key regulators of ECM assembly and cellular growth in animal cells (Iozzo, 1997; Iozzo *et al.*, 1999). The structure of the SHY protein is nearly identical to the structures of SLRPs and of the polygalacturonase inhibitor proteins (PGIP), plant cell wall glycoproteins capable of inhibiting fungal endo-polygalacturonases (PG). PGIPs are classified as defense proteins because their activity increases in response to wounding, elicitors, and fungal infection (De Lorenzo *et al.*, 2001). In higher plants, LRR-kinases have roles in development (Torii and Clark, 2000), host–pathogen interactions (Jones and Jones, 1997), phytohormone perception (Li and Chory, 1997), and pollen tube growth (Tang *et al.*, 2002). The extracellular domains (ECD) of many receptor-like kinases (RLKs) have a number of LRRs that function as interaction sites for signaling complexes (Torii and Clark, 2000). The tomato ortholog of SHY was shown to interact, in a yeast two-hybrid assay (Table S1, Tang *et al.*, 2002), and via an *in vitro* pulldown assay (Tang *et al.*, 2004) with the extracellular domain of LePRK2, a pollen-specific receptor protein kinase from tomato.

The increased level of *SHY* mRNA upon pollen germination, the predicted ECM localization of SHY, and the interaction of the tomato ortholog with a pollen-specific protein kinase suggested that SHY might play an important role during pollination. To test this hypothesis, we generated transgenic plants expressing an AS copy of the *SHY* cDNA in pollen. Using a combination of genetic and histochemical analyses, we show that pollen from AS-*SHY* plants exhibits severely altered germination and growth during pollination.

Results

SHY *is a single-copy gene in* Petunia hybrida

Numerous LRR proteins are encoded in plant genomes (Li *et al.*, 2003). To determine the copy number of *SHY*, restriction digests of genomic DNA were hybridized with two different probes: the 1357-bp full-length cDNA and an LRR-specific probe consisting of the *Sac*I/*Not*I fragment of the *SHY* cDNA (Figure 1b). Figure 1(a) shows a single fragment hybridized to the *SHY* cDNA probe in five different restriction enzyme digests of petunia DNA. An identical pattern of hybridization was obtained with the LRR probe, even when the blots were washed at low stringency (data not shown). Although other LRR proteins such as Pex1 (Rubinstein *et al.*, 1995), PRK1 (Mu *et al.*, 1994), and LePRK1, LePRK2 (Muschietti *et al.*, 1998) and LePRK3 (Kim *et al.*, 2002) are expressed in pollen, this result indicates that the *SHY*-LRR probe is specific for the *SHY* gene, and thus it seemed likely that an AS construct would only affect the expression of *SHY*. Database analyses indicate that the *SHY* sequence is conserved in many plants, including *Arabidopsis*, *Zea mays*, and many Solanaceae.

Structural features of the SHY *gene*

A 14.2-kbp lambda clone containing the genomic copy of *SHY* (g*SHY*) was recovered from a W37 inbred *P. hybrida* library. Figure 1(b) shows a map of the sequenced 3254-bp fragment, which contains the entire coding region as well as 1.96-kbp of 5′ flanking sequence, including a 336-bp UTR (GenBank accession number AF325673). There are no introns in the coding region of the *SHY* gene; a single intron is located in the 5′ UTR between nucleotides +152 and +328. A single transcription start site (+1) was detected 336-bp upstream of the putative translation start (Figure 1b). A search of the Plant Cis-Acting Regulatory DNA Elements (PLACE) database (Higo *et al.*, 1999) detected multiple copies of regulatory motifs which are crucial for pollen expression, including the PB core motif, TGTGGTT, a pollen-specific sequence found in *LAT*52 (Twell *et al.*, 1990), and a positive regulatory element, AGAAA, responsible for late pollen-specific activation (Bate and Twell, 1998). Several binding sites for MYB transcription factors were located in the g*SHY* 5′ sequence; they may function to mediate flavonoid induction of *SHY* expression (Solano *et al.*, 1995).

Comparison of the amino acid sequence of SHY with PGIPs and related proteins

The deduced SHY peptide is 353 amino acids with a putative 21 amino acid signal peptide (Figure 1c, domain A). The deduced molecular mass of the mature SHY protein is 35.9 kDa, with a predicted pI of 6.71. SHY contains four potential *N*-glycosylation sites and six potential *N*-myristylation sites. The 10 LRRs constitute the bulk of the SHY protein, starting at residue 105 and extending to residue 348 (Figures 2 and 3). Alignment of the LRRs (Figure 1c, domain C) reveals a 24 amino acid repeat that matches the e (external) LRR consensus, LxxLxxLxxLxLxxNxLxGxIPxx, as found in the tomato *Cf*-2 and *Cf*-9 genes (Jones and Jones, 1997).

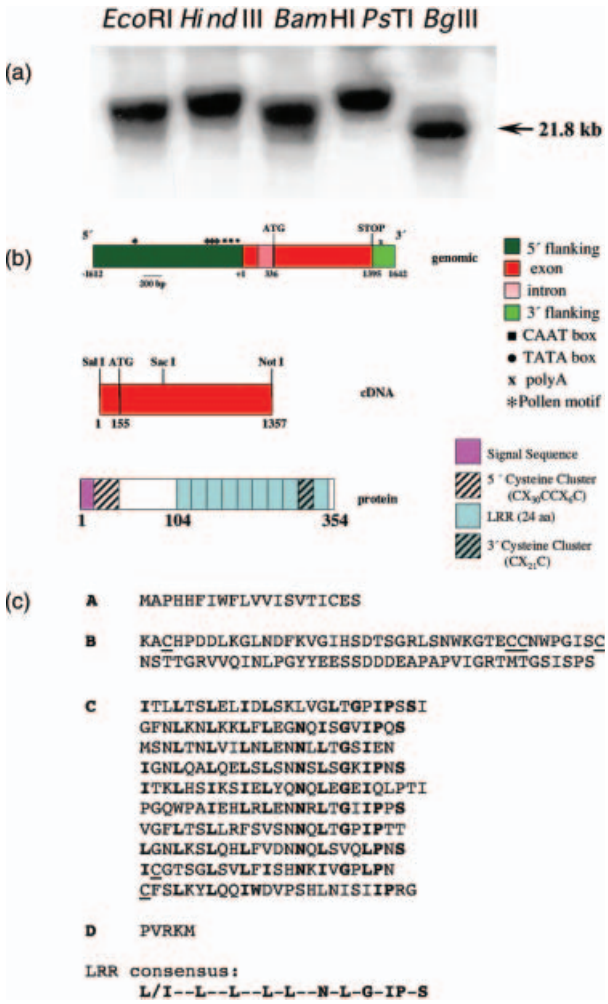

Figure 1. Structural features and organization of the petunia *SHY* gene.
(a) Southern analysis shows that *SHY* is a single copy gene. Restriction digest analysis of 10 μg of *Petunia hybrida* genomic DNA cut with *Eco*RI, *Hin*dIII, *Bam*HI, *Pst*I and *Bgl*II restriction enzymes and hybridized to a ^{32}[P]-labeled *SHY* cDNA.
(b) Diagram of the genomic, cDNA and protein sequence of *SHY*. The nucleotide sequence of g*SHY* has been submitted to Genbank, accession number AF325673.
(c) Primary structure of the SHY protein. The predicted amino acid sequence of SHY is shown divided into four domains (A–D). Domain A is a putative signal peptide of 21 amino acids. Domain B shows the N terminal cysteine cluster underlined. Domain C shows the 10 LRR repeats, with amino acids present at least in 50% of the repeats highlighted in bold. The consensus below is derived from alignment of the 10 LRR motifs.

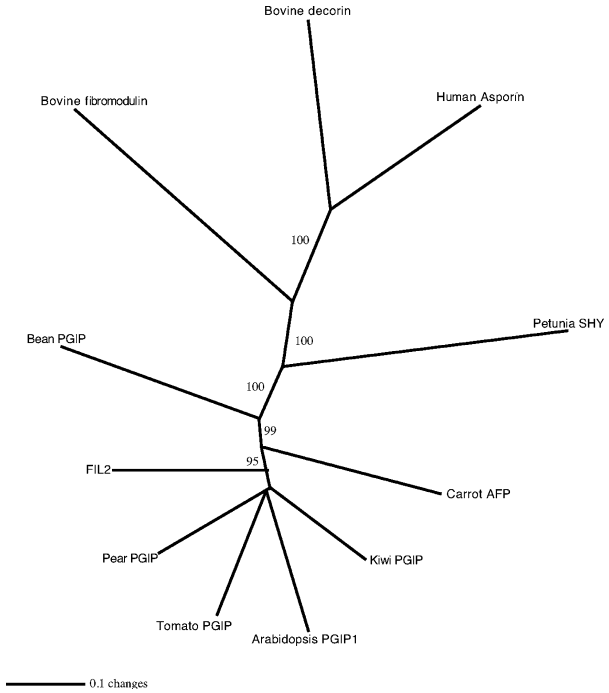

Figure 2. Phylogenetic comparison of SHY, PGIP, and SLRP proteins.
Petunia SHY and PGIP sequences from pear, tomato, kiwi, *Arabidopsis*, bean, carrot AFP, FIL2, and three SLRPs: human asporin and bovine decorin and fibromodulin were used to construct the unrooted tree.

The four-domain organization (Figure 1c) of the deduced SHY protein is nearly identical to the domain organizations of PGIPs of plants and of the SLRPs in mammals. SHY has an N-terminal cysteine-rich cluster (Figure 1c, domain B) that conforms to the PGIP-consensus sequence $Cx_{29-30}CCx_{5-6}C$ (Mattei *et al.*, 2001), but the C-terminal region lacks the $GGx_{12}CLCGxPL$ sequence that is considered a structural hallmark for the PGIP family (De Lorenzo *et al.*, 2001). Instead SHY has an SLRP-like C-terminus with two cysteines separated by 20–30 amino acids (Iozzo, 1997, 1999). Using the GenomeNet CLUSTALW, a multiple sequence alignment was obtained for 11 accessions of the SHY/PGIP/SLRP gene family. The neighbor-joining analysis resulted in the single unrooted tree shown in Figure 2. The neighbor-joining bootstrap analysis supports the placement of the petunia SHY protein between the PGIPs (bs = 100%) and the SLRPs (bs = 100%).

A SHY::eGFP fusion protein accumulates in the extracellular matrix

The structural similarity of SHY with other ECM proteins, and the finding that the SHY ortholog in tomato interacts with extracellular domain of a receptor kinase (Tang *et al.*, 2002, 2004) suggested that SHY might be located in the cell wall. To investigate its cellular location, a translational fusion was made of the entire *SHY* cDNA to the N-terminus of eGFP, under the control of the constitutive cauliflower mosaic virus 35S promoter. The eGFP fusion construct was introduced into onion epidermal cells using particle-mediated DNA delivery and the resulting eGFP fluorescent signal was visualized microscopically (Scott *et al.*, 1999). Figure 3 shows that, in contrast to the overall diffuse fluorescence of the eGFP control, the SHY::eGFP signal was observed mainly at the cell surface. When the tissues were plasmolyzed with

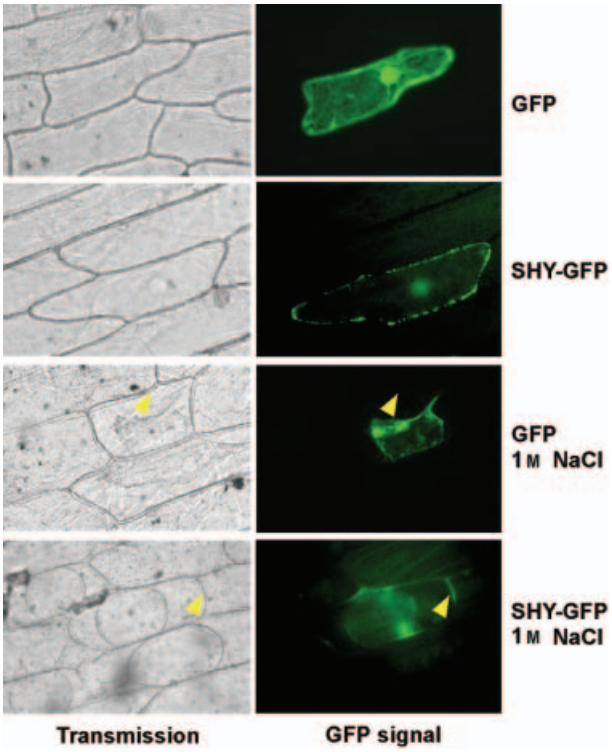

Figure 3. An SHY::eGFP fusion protein localizes to the cell wall. 35S::eGFP or a 35S::SHY::eGFP fusion protein after microprojectile-mediated DNA introduction into onion epidermal cells. To confirm cell wall localization, the bombarded tissue was plasmolyzed in 1 M NaCl and re-examined by epifluorescence (lower panels).

1 M NaCl most of the SHY::eGFP signal remained at the cell surface whereas the eGFP signal did not. This result confirms that SHY is directed to the cell wall, a location it presumably occupies in pollen tubes.

Transgenic plants expressing an antisense SHY *cDNA in pollen show reduced levels of* SHY *RNA and abnormal germination and growth* in vitro

A reverse genetic approach was used to test the function of SHY in pollination. We used the *LAT52* promoter from tomato (Twell *et al.*, 1990) to express an AS copy of the *SHY* cDNA in petunia pollen. We first determined that the *LAT52* promoter would express *SHY* RNA at the appropriate stages, by generating transgenic plants expressing an *LAT52-GUS* construct in petunia (data not shown). No activity was detected before microspore mitosis but, beginning at about stage 4 (Pollak *et al.*, 1993), GUS-positive pollen was detected. GUS activity increased with maturity and the highest levels of activity were measured in mature and germinating pollen. This is the exact same pattern of *SHY* RNA accumulation in the V26 background (Guyon *et al.*, 2000), and we therefore concluded that the *LAT52* promoter would be suitable for these experiments.

The *Sac*I/*Not*I fragment of the *SHY* cDNA (Figure 1b), which encodes most of the LRRs, was inserted in an AS orientation between the *LAT52* promoter and the *NOS* 3′ terminator in the SLJ7292 binary vector (Jones *et al.*, 1992) and introduced into petunia by *Agrobacterium*-mediated transformation. We reasoned that it was unlikely that AS expression of the *SHY LRR* domain would inhibit other pollen-expressed LRR proteins because Southern analysis (Figure 1a) showed that the *SHY-LRR* probe hybridized only with the *SHY* sequence. Forty-five primary transformants (T_0) were recovered after kanamycin selection and the presence of the transgene was confirmed by PCR amplification of the *NPT*II gene. Any effects of *LAT52*-driven expression of the AS-*SHY* construct would be expected to be evident late in pollen development, and to affect approximately 50% of the pollen grains.

Mature pollen from all individual T_0 plants was examined microscopically for altered morphology, viability (by fluorescein diacetate (FDA) staining), and *in vitro* germination frequency. The majority of primary transformants (38/45) produced viable pollen that was morphologically normal but that showed alterations in the frequency of *in vitro* germination and/or the rate of pollen tube growth when compared with pollen germination and growth rates of wildtype, V26 (data not shown). Pollen from the remaining seven T_0 transformants either showed no differences relative to V26 (five of 45) or was non-viable at maturity (two of 45). Next, to determine whether the mutation affected the pollen at earlier stages, anthers from three lines encompassing the broadest range of phenotypes were harvested at three different developmental stages. The fixed, sectioned and stained anthers were examined microscopically and with the exception of a line producing non-viable pollen (see above) no developmental defects were detected (data not shown).

The 38 plants producing viable pollen that showed some deviation from the normal *in vitro* germination frequency or tube growth rate were tested by RNA gel blot analysis to determine whether the level of expression was reduced. A representative blot (Figure 6a) using total pollen RNA and the *SHY* cDNA as a hybridization probe (Guyon *et al.*, 2000) shows that the transcript level in most T_0 transformants was reduced relative to the level seen in the V26 parent, as was observed in AS-*LAT52* transgenic plants (Muschietti et al. 1994). Expression analyses with the T_1 and T_2 generations are discussed below.

Genetic analysis

The T_0 plants were self-crossed to determine transmission ratios and to generate homozygous AS-*SHY* lines. Most of the self-crosses resulted in capsules that contained a reduced seed number compared with V26 self-crosses, i.e. 50–110 seeds instead of approximately 225 seeds. Transmission of the AS-*SHY* gene was monitored by ger-

minating the T_1 or T_2 seeds on medium supplemented with kanamycin (Kan). Mendelian segregation during self-crosses predicts a 3:1 ratio of Kan^R:Kan^S for a single transgene insertion. However, if *SHY* expression is required for the pollen to function, the transgene will not transmit through the pollen and a 1:1 ratio of Kan^R:Kan^S progeny is predicted. Most of the T_1 transformants showed a 2:1 Kan^R:Kan^S ratio, suggesting that transmission of the transgene might be impaired. Crosses of Kan^R T_1 or subsequent generation plants (see Table S1) as female with V26 pollen resulted in full seed capsules, demonstrating that female fertility was not affected in the AS-*SHY* plants. Thus we concluded that the altered segregation ratio likely resulted from impaired transmission of the AS-*SHY* pollen. Southern analysis was used to confirm the presence of, and to assess the copy number of, the AS construct in those Kan^R plants for which T_1 and T_2 generations were obtained. Of the primary transformants tested in this manner, most contained one copy of the transgene, but a few plants harbored two copies (data not shown).

Nine individual single copy lines that showed a moderate to substantial reduction in *SHY* RNA in the T_0 generation (Figure 6a) were repeatedly self-crossed (Table S1) and eventually sufficient T_1 and T_2 generation seed was obtained to be able to identify plants homozygous for the AS-*SHY* transgene. The genotype was confirmed by germinating the contents of a seed capsule on Kan; only those plants that produced 100% Kan^R progeny were scored as homozygous for the transgene. It was difficult to obtain seed from outcrosses of homozygous (T_1 and/or T_2) AS-*SHY* pollen to V26 stigmas (e.g. four capsules in 34 attempts) and the number of progeny was severely reduced, with an average of 20 seeds per capsule. These results support the conclusion that only a small fraction of the AS-*SHY* pollen competes successfully in fertilization.

AS-SHY *pollen shows severely impaired pollen–pistil interactions that correlate with reduced* SHY *RNA levels*

We noticed that the impaired *in vitro* pollen germination phenotype was more striking in the T_1 and T_2 generations. For example, in the T_0 generation both AS-*SHY* 57.2 and AS-*SHY* 41.1 pollen germination was scored at a slightly reduced frequency (approximately 45%) compared with wildtype (approximately 65%) (not shown). However, a detailed analysis of the *in vitro* germination frequency and subsequent pollen tube growth rate for the T_2 generation of AS-*SHY* 57.2 and AS-*SHY* 41.1, two lines that were homozygous for the transgene, revealed a more significant phenotype. Compared with V26 germination (Figure 4a), only 1% of the T_2 57.2.3D pollen grains germinated after 2 h of incubation in GM (Figure 4b). Because this frequency rose to 20% after 24 h, we concluded that the pollen remained viable but that the initiation of germination was severely retarded. Germination of T_2 41.1.8C pollen was relatively high at 2 h (37%) but the subsequent tube growth rate appeared slower (Figure 4c) than in wildtype, an observation that was confirmed by the time course analysis shown in Figure 4(d).

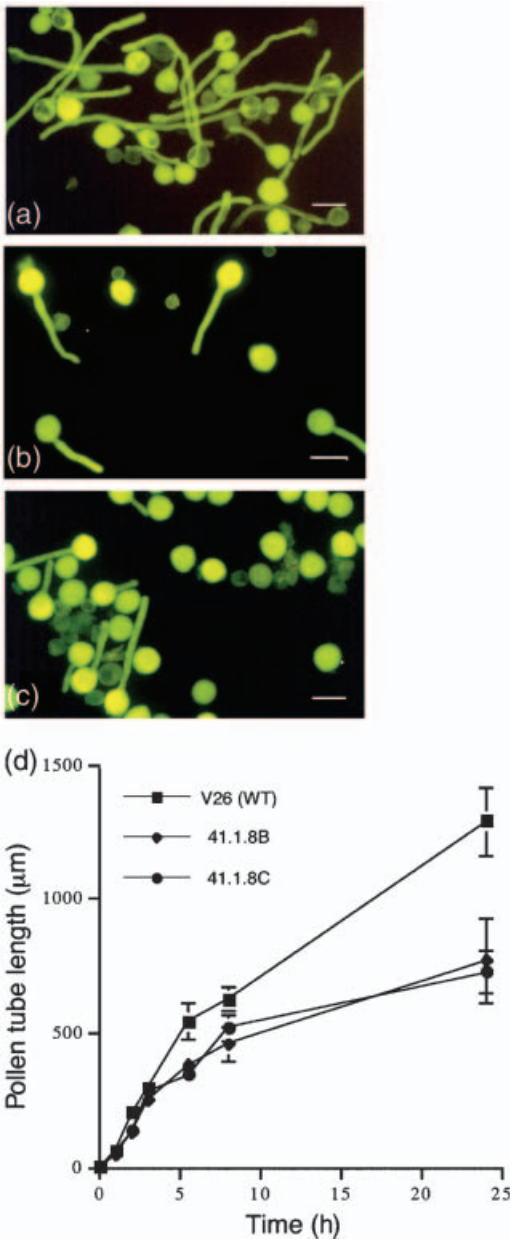

Figure 4. Viability, germination, and tube growth rate of AS-*SHY* pollen.
Fluorochromatic test after *in vitro* germination of AS-*SHY* pollen. Pollen from untransformed V26 plant (a), and AS-SHY plants 57.2.3D (b) and 41.1.8C (c) were germinated *in vitro* for 2 h, stained with FDA, and visualized by epifluorescence. Bar = 50 μm.
(d) Pollen tube growth rate of T_2 generation pollen.

The *in vitro* germination phenotype of the AS-*SHY* pollen showed some abnormalities but these defects did not seem severe enough to account for difficulty in obtaining seed from the self- and out-crosses. Successful

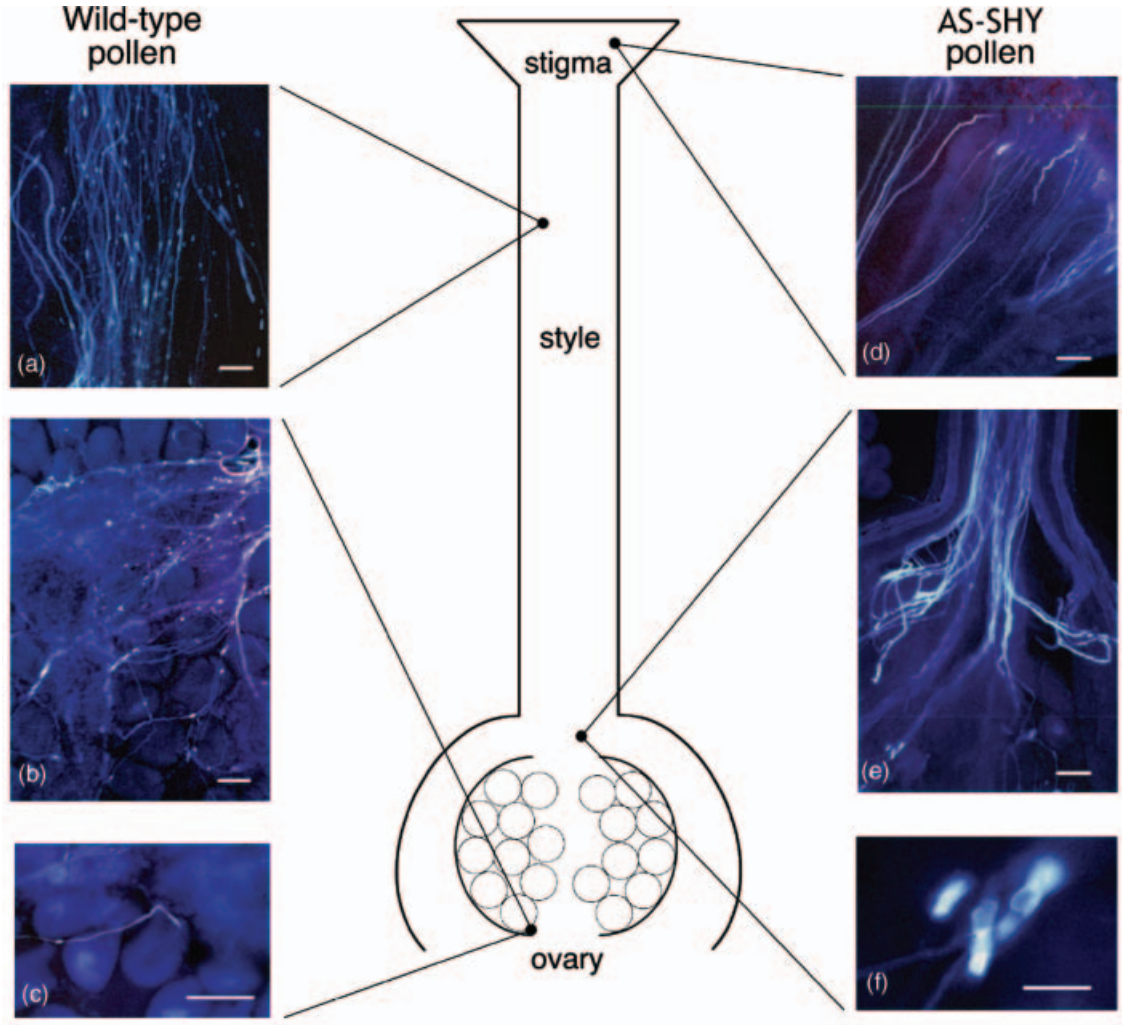

Figure 5. *In vivo* pollen germination assays.
V26 pistils self-crossed (a–c) and cross-pollinated with 57.2 AS-*SHY* pollen (d–f) and harvested 60 h post-pollination. Pistils were stained with decolorized aniline blue and visualized by epifluorescence. Bar in a–e = 200 μm; in f = 50 μm.

fertilization depends on a series of interactions between pollen and pistil factors in a complex and changing environment that the *in vitro* germination and growth assay cannot replicate (Lord and Russell, 2002; Palanivelu *et al.*, 2003). To determine whether the *in vivo* behavior was more severely impaired, selected AS-*SHY* plants from the T_1 and/or T_2 generations were examined by performing limited pollinations onto wildtype (V26) pistils. The germination and growth of individual tubes on the path from stigma to ovary was followed by visualization of callose deposits (Vogt *et al.*, 1994). For reference we documented the growth of the parental V26 pollen in self-pollinated pistils, wherein germination occurs within 30 min of application to the stigma and by 90 min post-pollination the tube tips have penetrated deep into the stigma. At 8 h the growing front of tubes has passed the stigma–style interface (Figure 5a) and fertilization occurs approximately 48 h after pollination, after the tip enters the micropyle (Figure 5b,c). In contrast, pollen germination is delayed in the 57.2.3D AS-*SHY* plants (although most germinated within 90 min) and after 8 h of incubation, only a few AS-*SHY* pollen tips are beyond the stigma–style interface (Figure 5d). At 48 h post-pollination most AS-*SHY* tubes are still within the style. At 60 h post-pollination, 95% of the transgenic pollen grains still have not entered the ovary, although a few have reached the top one to three ovules and an even smaller number show evidence of entry into the micropyle (Figure 5e). Tube growth of the AS-*SHY* pollen was monitored for 120 h but no further progression beyond the top of the ovary occurred, for the majority of the tubes. Moreover, the transgenic pollen tubes typically did not show periodic deposits of callose plugs and instead accumulated dense, streaky callose throughout the length of the tube (Figure 5d,e). In addi-

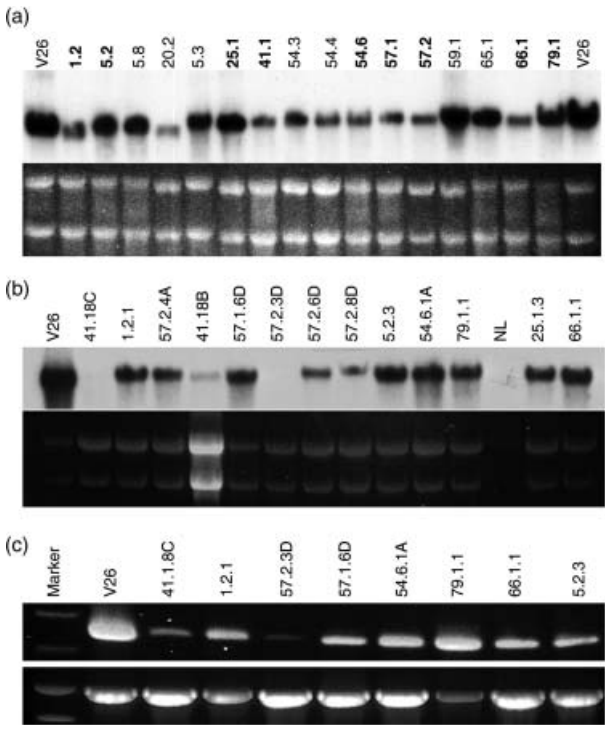

Figure 6. Expression analysis of AS-*SHY* pollen.
(a) RNA gel blot analysis of *SHY* transcripts in 16 T_0 plants and the V26 parental line. An ethidium bromide-stained gel shows the rRNA levels in each sample. The nine T_0 lines labeled in bold were selected for further analysis.
(b) RNA gel blot analysis of *SHY* transcripts from 13 T_1 or T_2 plants homozygous for the AS-*SHY* transgene. NL, not loaded. Lower panel is an ethidium bromide-stained gel showing the rRNA levels.
(c) RT-PCR analysis of *SHY* transcripts from homozygous AS-*SHY* pollen. Total pollen RNA was isolated from T_1 and T_2 plants and the untransformed parental line (V26) and used as template in RT-PCR reactions with either *SHY*-specific primers (top panel) or with actin-specific primers (bottom panel). A correlation was observed between the most severely affected fertilization rate and the greatest decrease in *SHY* RNA levels. The bottom panel shows that there was no significant difference in the amount of actin-specific sequences in the various samples.

tion, the tubes that arrested in the upper part of the ovary had swollen tips with large callose accretions (Figure 5f).

To determine whether the *SHY* phenotype correlated with a decrease in *SHY* transcripts, RNA gel blot analysis was performed on the nine independent homozygous lines. The T_1 and/or T_2 generation AS-*SHY* pollen accumulated significantly reduced amounts of *SHY* RNA compared with the V26 parent and two lines, 41.1.8C and 57.2.3D, accumulated no detectable *SHY* RNA by this analysis (Figure 6b). To specifically detect endogenous transcripts (Gupta *et al.*, 2002), we performed RT-PCR using 5′ and 3′ primers that were outside of the sequences present in the AS-*SHY* construct. As shown in Figure 6 (c), almost all of the AS plants tested showed a reduction in *SHY* mRNA in pollen compared with the level detected in the V26 parent. Moreover, the increased sensitivity of the RT-PCR procedure detected very low levels of *SHY* RNA in the 41.1.8C and 57.2.3D plants.

AS-SHY *pollen is not impaired in PGIP activity*

The deduced SHY peptide shares sequence similarity with PGIPs, proteins that function as defense proteins by inhibiting fungal endo-PGs. To determine whether SHY might function as a PGIP, pollen extracts from wildtype (V26) and AS-*SHY* 57.2.3D, in which the endogenous *SHY* transcript was dramatically reduced (Figure 6), were tested for PGIP protein and activity. A Western blot probed with a rabbit polyclonal antibody prepared against a *Phaseolus vulgaris* PGIP detected a 40-kDa doublet, the expected size for PGIP, in pollen extracts from both wildtype and transgenic pollen (data not shown).

PGIP activity was measured *in vitro* using a polygalacturonic acid-agarose plate PG inhibition assay, with PGs from *Aspergillus niger*, *Fusarium moniliforme*, *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani*, *Colletotricum lindemuthianum*, *Stenocarpella maydis*, and *Botrytis cinerea* as test substrates (Table 1). Comparable percent-

Table 1 Inhibitory activity of protein extracts from wildtype and AS-*SHY* pollen against fungal polygalacturonase (PG)

	% Inhibition					
	V26 (wildtype) pollen[a]			AS-*SHY* pollen[a]		
PG[b]	45 μg	4 μg	2 μg	45 μg	4 μg	2 μg
Aspergillus niger	16	nt	nt	21	nt	nt
Fusarium moniliforme	0	nt	nt	0	nt	nt
Fusarium oxysporum	0	nt	nt	0	nt	nt
Rhizoctonia solani	0	nt	nt	0	nt	nt
Colletotrichum sp.	100	100	72	100	100	72
Stenocarpella maydis	100	86	36	100	85	36
Botrytis cinerea	100	25	19	100	26	18

nt, not tested.
[a]Different amounts of total pollen proteins were tested for inhibitory activity against different fungal PGs.
[b]One agarose diffusion unit, as described by Ferrari *et al.* (2003) of each PG was used.

ages of inhibition of PG were observed with dialyzed protein extracted from wildtype and transgenic pollen. These results indicate that PGIP activity exists in mature petunia pollen and that this activity is not reduced in AS-*SHY* 57.2.3D pollen. We conclude that either SHY is not a PGIP or is not active against the fungal enzymes tested. Thus the abnormal pollen phenotype cannot be attributed to a loss of PGIP activity.

Discussion

Several lines of evidence suggest that the SHY gene product functions during pollination and is required for successful fertilization. Reducing or eliminating the *SHY* transcript by AS expression produced morphologically normal and viable pollen but its efficiency in *in vitro* pollen germination was reduced relative to that of wildtype pollen. Among the 38 T_0 lines that showed a reduction in *SHY* RNA and/or an altered germination frequency compared with wildtype, we selected nine lines for detailed analyses. These nine lines showed significantly reduced seed set in the T_1 and T_2 generations in self-crosses, and pistil squashes in the T_1 and T_2 generation showed that the pollen tube growth rate was reduced, relative to seed set and pollen tube growth rate in wildtype.

Repeated attempts were required to obtain homozygous T_1 and T_2 generation seed. To determine the basis of the reduced seed set, we analyzed pollen from affected plants during limited pollinations to wildtype pistils. Although both germination and tube growth were retarded *in vivo*, most tube tips eventually reached the apex of the ovary; but then either growth ceased or was arrested in the majority of tubes. As a result, few ovules were fertilized and seed production was reduced by more than 95% in some lines. Within the pistil the AS-*SHY* pollen tubes showed an increased amount, and an altered pattern, of callose deposition. Abnormal deposits in the growing and arrested tubes indicate impaired pollen–pistil interactions (Geitmann *et al.*, 1995 and references therein) but virtually nothing is known about the factors that regulate callose plug formation (Lord, 2000). We speculate that the reduced seed set observed in the T_0 generation was a reflection of two factors: the failure of most AS-*SHY* pollen to reach the ovary and that fewer wildtype pollen tubes were able to reach ovules because of interference in the pistil and upper ovary from the abnormal callose deposits generated by the AS-*SHY* tubes. Abnormal callose patterning, including large deposits in the tip, was associated with reduced male transmission of pollen carrying mutations in the GPI-anchor biosynthetic genes, *SETH1* and *SETH2* (Lalanne *et al.*, 2004) and, like the AS-*SHY* pollen, the reduction in pollen germination frequency and tube growth rate was more severely manifested *in vivo* than *in vitro*. The major difference was that *SETH* pollen failed at germination and/or early tube growth on the stigma whereas the failure of the AS-*SHY* tubes occurred at the ovary apex, just before contacting the ovules.

The structural and sequence similarity of SHY with SLRPs and PGIPs is striking. Both SLRPs and PGIPs are localized to the ECM and a similar location was confirmed for SHY by transiently expressing an eGFP fusion construct. However, the structural similarity does not translate into obvious functional homology with respect to PGIP activity. The difficulty in assigning a functional role to similar proteins is illustrated by carrot AFP, which has antifreeze activity (Worrall *et al.*, 1998) but is classified as a PGIP based on sequence homology (Jones and Jones, 1997). In the absence of testing the purified protein against every conceivable PG substrate, Worrall *et al.* (1998) concluded that the phylogenic distance supported the conclusion that AFP was not a PGIP. By the same token, the enzyme analysis (Table 1), that indicates that SHY does not have PGIP activity, and the phylogram (Figure 4), which shows that SHY is as related to the mammalian SLRPs as it is to AFP, support our conclusion that SHY is not a PGIP. The corollary is that PGIP activity is not responsible for the mutant phenotype.

SLRPs are ECM proteins that mediate matrix homeostasis and cell proliferation. Decorin is the best characterized SLRP and its multiple roles are all mediated through the LRR sequences (Iozzo, 1999). Decorin is involved in collagen assembly and ECM remodeling (Iozzo, 1997; Yamaguchi *et al.*, 1990) and in cell proliferation via its ability to bind to the EGF receptor kinase and trigger a signal cascade (Iozzo *et al.*, 1999). Decorin regulates cell proliferation by binding transforming growth factor-β (TGF-β), the major signal peptide regulating growth and differentiation in animal cells, thus preventing its interaction with the TGF-β receptor (Iozzo, 1999).

The LRR is an ancient and highly versatile protein-binding motif and SHY consists almost entirely of LRRs and has no other recognizable signaling motifs. Thus it is logical to assume that any interactions of SHY with pistil factors or pollen proteins are mediated by the LRRs. RLKs are the largest family of LRR proteins in pollen and most have an ECD with five to six tandem eLRRs, a membrane spanning region and a cytoplasmic kinase domain with ser/thr specificity (Kim *et al.*, 2002; Torii and Clark, 2000). Although the exact mechanism has to be elucidated, there is good evidence that PRKs are involved in pollination (Muschietti *et al.*, 1998). The finding that the tomato ortholog of SHY binds to the extracellular domain of a pollen receptor kinase (Tang *et al.*, 2002, 2004) suggests that SHY functions in cell–cell communication to modulate pollen growth.

At all stages growth relies on cell surface interactions between pollen and pistil proteins (Lush, 1999; Palanivelu

and Preuss, 2000). Pollen–stigma interactions involve recognition, adhesion and hydration, and small molecules, such as water, lipids and flavonoids, are important for pollen germination (reviewed in Taylor and Hepler, 1997). Most pollen–style interactions occur within the nutrient-rich ECM of the transmitting tract cells, and specific molecules within the stylar matrix that promote pollen tube growth include various arabinogalactan proteins (AGPs) such as the *N. tabacum* transmitting tract-specific AGP (TTS) and a 120-kDa AGP from *N. alata* (Lind *et al.*, 1996; Wu *et al.*, 1995). Some growth-promoting molecules also function as guidance cues. TTS forms a glycosylation gradient within the style which may serve a nutritive or guidance role (Cheung, 1995). Chemocyanin, a small basic protein from the lily stigma, acts with a cysteine-rich adhesin to induce directional pollen tube growth *in vitro* (Kim *et al.*, 2003). In late stages of pollination, chemoattractants reorient pollen tube growth toward the ovules. An unidentified attractant emanating from the synergid cells was detected (Higashiyama *et al.*, 2001) and recently *Arabidopsis* pollen tube growth and guidance into the micropyle was shown to be regulated by a highly focused GABA gradient (Palanivelu *et al.*, 2003). Although the mutant AS-*SHY* phenotype is manifest at all stages of pollination, it culminates in tube arrest at the top of the ovary. The likely cell wall location of SHY, the increased severity of the AS-SHY phenotype during interactions with the pistil tissues, and the putative signaling and protein interaction properties of LRR proteins suggests that SHY, or a pollen complex containing SHY, facilitates growth through the pistil and ovary. This warrants a search for interacting partners of SHY from different regions of the pistil.

Experimental procedures

Plant material

V26 (wildtype) petunia plants were grown under standard greenhouse conditions, as well as *in vitro* to provide sterile material for transformation. T_0 and T_1 transformants were self- and cross-pollinated manually. Mature pollen was collected from individual anthers and used immediately for *in vitro* germination and cytological analyses. Because the V26 line can produce a significant number of aborted pollen grains (Taylor and Jorgensen, 1992), control and transformed plants of the same age and from the same greenhouse were scored together.

Isolation and sequence analysis of genomic clone and predicted protein

A W137 petunia genomic library cloned into the *Sac*I/*Xho*I half arm sites of phage λGEM11 was screened with the full length *SHY* cDNA as a probe using standard techniques (Sambrook *et al.*, 1989). Plaques (4.8×10^4) were screened under highly stringent conditions and one positive plaque was identified and purified through two additional rounds at low plating density. An *Sac*I digest released the 14.2 kbp insert as two fragments of 5.2 and 9 kbp. Southern hybridization was used to orient the fragments, i.e. the 5′ portion of the *SHY* cDNA was contained on the 5.2 kbp fragment. The fragments were individually subcloned into pBluescript SK+ (Stratagene, La Jolla, CA, USA) and plasmid DNA was prepared with High Pure Plasmid Isolation Kit (Boehringer Mannheim, Indianapolis, IN, USA). DNA sequencing was accomplished using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and extension products were analyzed with the Applied Biosystems DNA Sequencer, Applied Biosystems, Foster City, CA, USA. Sequences were analyzed using the GCG package (Devereux *et al.*, 1984) and the PLACE database (Higo *et al.*, 1999) (http://www.dna.affrc.go.jp/htdocs/place/signalscan.html).

A multiple sequence alignment using the ClustalW server: http://www.clustalw.genome.ad.jp/ was performed with the following protein sequences: PGIPs from pear (AAA33865), tomato (S47965), kiwi (CAA88846), bean (P35334), *Arabidopsis* (AF229249), carrot AFP (AAC62932), FIL2 (CAA54303), human asporin (AAK35161), bovine decorin (P21793) and fibromodulin (S05390), and petunia SHY (AF049920). The phylogenetic structure of this data set was explored using neighbor-joining methods, as implemented by PAUP*4.0b8a (Swofford, 2002; random tie breaking, mean character difference). Branch support was explored using the neighbor-joining bootstrap option in PAUP*4.0, with 10 000 replicates.

Start of transcription

The 5′ terminus of the *SHY* gene transcript was determined by primer extension (Sambrook *et al.*, 1989). A 21-mer oligonucleotide (AGCAATCCTAAAATCCACGAAC) was end-labeled with ^{32}P using polynucleotide kinase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Total RNA (50 μg) from pollen was annealed with the labeled oligonucleotide (10^5 cpm) for 12 h at 30°C in 30 ml of hybridization buffer (40 mM PIPES, 1 mM EDTA, 400 mM NaCl, 80% formamide). The ethanol-precipitated sample was resuspended in 30 μl of transcriptase buffer and the primer extension reaction was carried out at 37°C for 2 h with 50 units of SuperScript II Moloney Murine Leukemia virus reverse transcriptase (GIBCO-BRL, Bethesda, MD, USA) and 30 units of RNase inhibitor. After treatment with ribonucleases, phenol extraction and ethanol precipitation, the nucleic acids were resolved on a 6% polyacrylamide/8 M urea sequencing gel. A sequencing reaction (fmol DNA sequencing kit, Promega) using the same primer and the 5.2 kbp genomic subclone as a template provided the reference sequence. The gel was fixed, dried and results were visualized by autoradiography.

SHY localization

eGFP imaging in onion epidermal cells and cell walls was performed according to Scott *et al.* (1999). 35S::SHY::eGFP was constructed by inserting the full length *SHY* cDNA in an N-terminal fusion with the enhanced (e)GFP sequence in the pPK100 vector (a kind gift of Drs Robert Blanvillain and Patrick Gallois, University of Manchester, UK). DNA-coated gold particles (1.0 μm; Bio-Rad, Hercules, CA, USA) were bombarded into onion epidermal cells using a Biolistic PDS-100/He system (Bio-Rad) with 1100 psi rupture discs. After bombardment, the cells were allowed to recover for 18–22 h on 0.7% agar plates at 22–26°C in continual light. Onion peels were screened for eGFP fluorescence using an Olympus SZX12 dissecting microscope with a fluorescence attachment (Olympus, Tokyo, Japan). Peels with fluorescent cells were bathed in 20 mM piperazine-*N*-*N*′-bis (2-ethanesulfonic acid) (PIPES)-KOH (pH 7.0) for 6–12 h so that eGFP in the cell wall could be visualized, then plasmolyzed by incubation in

1 M NaCl. Fluorescence and light microscopy were performed using a Zeiss Axiophot compound microscope (Zeiss, Jena, Germany) and the images were captured digitally.

Construction of antisense gene and plant transformation

The AS-*SHY* vector was constructed by inserting an AS orientation of the *Sac*/*Not*I 0.87 kbp fragment of the *SHY* cDNA between the 0.6 kbp minimal LAT52 promoter and the 3′ NOS terminator by blunt-end ligation, after *Nco*I digestion removed the GUS gene from pLAT52-7 (Twell *et al.*, 1990). An *Sal*I/*Eco*RI fragment was then subcloned into the *Xho*I/*Eco*RI sites of the SLJ7292 binary vector (Jones *et al.*, 1992), obliterating both the *Xho*I and *Sal*I sites after ligation. The resulting construct was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating and *Petunia hybrida* leaf discs were transformed according to Napoli *et al.* (1999). Plantlets (T_0) emerging from callus were excised and transplanted onto SRM medium containing 100 μg ml^{-1} of kanamycin and then to soil after root formation.

PCR screening

Genomic DNA was extracted from frozen leaf disks according to Oard and Dronavalli (1992) and dissolved in 50 μl of TE pH 7.5. PCR amplification was performed on 2 μl of DNA in the presence of 1.5 mM of $MgCl_2$, using primers from the *LAT52* promoter and the 3′ *NOS* terminator and the following conditions: denaturation 1 min at 94°C, annealing 30 sec at 57°C, extension 90 sec at 72°C for 30 cycles, final extension for 5 min. One-fifth of the reaction was run onto an agarose gel to check for the presence of the transgene. DNA from the *Agrobacterium* transconjugant and from untransformed plant material was used as positive and negative controls, respectively.

Genetic analysis

T_0 plants were self-pollinated and seeds were placed onto BSM medium containing 100 μg ml^{-1} of kanamycin. Plantlets were scored for kanamycin resistance (true leaves and branched roots) versus sensitivity (only cotyledons and stunted roots). Genetic ratios were calculated as the percentage of resistant plants to the total number of plants. From each primary transformant, 12 kanamycin-resistant progeny were transferred to soil and grown to maturity. They were then allowed to self-pollinate and the seeds tested for kanamycin resistance to identify homozygotes.

Microscopic analysis

In vitro *pollen germination.* Stage 9 flowers (Pollak *et al.*, 1993) were collected and pollen was rapidly dispersed into PEG-based germination medium (GM) as described (Mo *et al.*, 1992). Germination was monitored after 2 h of *in vitro* incubation, for both transformed and the control wildtype plants. Percentage germination was normalized to account for aborted pollen grains. For the time course study, the length of at least 20 pollen tubes was measured after 1, 2, 3, 5.5, 8, and 24 h. After 24 h, the tubes were entangled and could no longer be measured. Data from three replicate experiments performed on different days were plotted with the SD.

Fluorochromatic test. Fluorescein diacetate was added to mature pollen grains in GM at 0.1% and allowed to penetrate the pollen grains for 15 min before observation (Heslop-Harrison *et al.*, 1984). Membrane integrity was checked under fluorescent microscope.

Decolorized aniline blue staining of pollinated pistils. Pistils were self- and cross-pollinated and pollen allowed to germinate for 1.5, 8, 30, 48, 60, 72, and 120 h. The pistils were harvested, alkaline-treated and stained with 0.1% decolorized aniline blue, as described in Vogt *et al.* (1994). Pistils were infiltrated in glycerol, mounted and visualized with a fluorescent microscope under blue light (emission at 410 nm).

RNA and DNA gel blots

RNA isolation and Northern analysis using 10 μg of total RNA was performed as described (Guyon *et al.*, 2000). Genomic DNA was extracted from young leaves using the CTAB method (Doyle, 1990). Approximately 10 μg of DNA was digested with restriction enzymes, separated on a 0.6% agarose gel and blotted onto nylon membrane using standard procedures (Sambrook *et al.*, 1989). The membranes were hybridized either with a ^{32}P-labeled random-primed *SHY* probe, or with a ^{32}P-labeled *Sac*/*Not*I fragment that comprises the LRR region of *SHY*.

RT-PCR analysis

Total pollen RNA from T_1 and T_2 AS-*SHY* plants was treated with DNase (TURBO DNA-free; Ambion, Foster City, CA, USA) to remove contaminating DNA. One microgram of RNA was subjected to RT-PCR (SuperScript One-Step RT-PCR with platinum *Taq*; Invitrogen, Carlsbad, CA, USA) using the following conditions: 30 min at 55°C following by 2 min at 94°C and 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, with a final 10 min extension at 72°C. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide under UV light. To ensure that only native transcripts would be detected, primers were designed to sequences outside of the cDNA region used in the AS construct. An actin control used the universal actin primers ACT119S and ACT245A and the cycling conditions described in McKinney *et al.* (1995).

Preparation and assays of PGs and PGIP

Homogeneous PGII of *A. niger*, homogeneous PG of *F. moniliforme* expressed in *Saccharomyces cerevisiae*, and a crude PG preparation of *F. oxysporum* f. sp. *lycopersici* were as described in Ferrari *et al.* (2003). Crude PG preparations were obtained from *R. solani*, *S. maydis* and *Colletotrichum* sp., after growth in Czapek medium (2 g l^{-1} NaNO3, 1 g l^{-1} K2HPO4, 0.5 g l^{-1} MgSO4, 0.5 g l^{-1} KCl, and 10 mg l^{-1} FeSO4, pH 7.0) containing 1% pectin from citrus fruit (Sigma, St. Louis, MO, USA) on a rotary shaker at 22°C for 2 weeks. Crude PG was obtained from *B. cinerea* grown in Czapek medium containing 1% glucose on a rotary shaker at 22°C for 2 weeks. Pollen was extracted from V26 and AS-*SHY* 57.2.3D plants. Pollen was recovered by centrifugation at 2500 ***g*** for 10 min. The pellet was homogenized in liquid nitrogen and resuspended in 1 M NaCl, 20 mM sodium acetate, pH 4.7 in the presence of protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 8000 ***g*** for 20 min and the supernatant dialyzed against 50 mM Na acetate pH 5.0, 100 mM NaCl. No endogenous PG activity was detected in the protein extracts after dialysis. Samples were subjected to SDS-PAGE and Western blot analysis,

and tested for inhibitory activity against fungal PGs. Two independent experiments were performed, and each extract was analyzed at least twice. PGIP activity in pollen protein extracts was inactivated by treating at 80°C for 10 min. PGIP activities were determined by radial diffusion assay, using 1 agarose diffusion unit of each PG, as described by Ferrari *et al.* (2003).

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2162/TPJ2162sm.htm

Table S1 Fertility data on self- and out-crosses of AS-*SHY* plants analyzed in this study. S, capsules containing <20 seeds; M, 20–100 seeds; L, >100 seeds. S capsules only gave rise to S capsules in subsequent generations. M capsules gave rise to S or M capsules in subsequent generations

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