# PCP-A1, a Defensin-like Brassica Pollen Coat Protein That Binds the *S* Locus Glycoprotein, Is the Product of Gametophytic Gene Expression

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Self-incompatibility (SI) in *Brassica* species is controlled by a single polymorphic locus (*S*) with multiple specificities. Two stigmatically expressed genes that have been cloned from this region encode the *S* locus glycoprotein (SLG) and *S* receptor kinase (SRK). Both appear to be essential for the operation of SI. It is believed that rejection of incompatible pollen grains is effected by recognition events between an as yet unidentified *S* locus–encoded pollen coating–borne protein and the SLG/SRK. We previously identified a small pollen coat protein PCP7 (renamed here PCP-A1, for pollen coat protein, class A, 1) that binds with high affinity to SLGs irrespective of *S* genotype. Here, we report the cloning of *PCP-A1* from *Brassica oleracea* and demonstrate that it is unlinked to the *S* locus. In situ localization of *PCP-A1* transcripts revealed that they accumulate specifically in pollen at the late binucleate/trinucleate stage of development rather than in the tapetum, which previously was taken to be the principal source of the pollen coat. PCP-A1 is characterized by the presence of a structurally important motif consisting of eight cysteine residues shared by the plant defensins. Based on the presence of this motif and other data, homology modeling has been used to produce a putative structure for PCP-A1. Protein–protein interaction analyses demonstrate that SLG exists in monomeric and dimeric forms, both of which bind PCP-A1. Evidence is also presented for the existence of putative membrane-associated PCP-A1 binding proteins in stigmatic tissue.

# INTRODUCTION

Flowering plants have evolved a range of both morphological and molecular mechanisms that prevent self-pollination. It has been estimated that up to 50% of angiosperms possess such self-incompatibility (SI) systems (Darlington and Mather, 1949; Brewbaker, 1959), highlighting their evolutionary importance (Stebbins, 1950, 1957; Whitehouse, 1950; de Nettancourt, 1977). The most advanced SI systems are thought to be those that are controlled by a single multiallelic locus (S locus), typified by Brassica, Nicotiana, and Papaver spp. In Nicotiana and Papaver spp, in which pollen compatibility is controlled by the gametophyte, the grain is rejected if the S allele it carries is shared by the stigma. Importantly, pollen compatibility in Brassica spp is regulated by the sporophyte, and thus the S alleles of the pollen parent determine whether the grain is accepted or rejected. In recent years, advances have been made toward elucidating the molecular basis of SI in these and other species (reviewed in Franklin et al., 1995; Hiscock et al., 1996; Kao and McCubbin, 1996).

The Brassica *S* locus has been estimated to span several hundred kilobases of DNA (Boyes and Nasrallah, 1993). To date, two polymorphic stigmatically expressed *S* locus genes that appear to be required for the operation of SI have been cloned; one encodes a transmembrane receptor-like protein kinase (Stein et al., 1991) termed SRK (for *S* receptor kinase), and the other encodes a glycoprotein (Nasrallah et al., 1985) termed SLG (for *S* locus glycoprotein). Plants with mutated *SRK* genes fail to reject "self"-pollen (Goring et al., 1993; Nasrallah et al., 1994; Stahl et al., 1998), as do plants in which *SLG* transcripts are downregulated (Nasrallah et al., 1992; Shiba et al., 1995).

The SRK is targeted to the plasma membrane of the papillar cells that cover the stigma surface (Delorme et al., 1995). It is envisaged that after self-pollination, the SRK is activated by binding a pollen-borne *S* ligand, which triggers a chain of intracellular signaling events that ultimately leads to pollen rejection (Nasrallah and Nasrallah, 1993). The *SLG*, which has striking sequence homology with the putative

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receptor domain of the SRK ( $\sim$ 90%), is also expressed in the papillar cells, with the mature protein being secreted into the cell wall. It has been suggested that maintenance of this homology is crucial for operation of the SI system (Nasrallah and Nasrallah, 1993). Interestingly, recent comparative studies of the hypervariable domains of these proteins, which are presumed to encode *S* specificity, have revealed that they are not always highly conserved between the SLG and SRK of the same haplotype and may therefore bind different domains of the male *S* determinant (Kusaba et al., 1997).

Although substantial progress has been made in identifying and characterizing the female components of the SI system in Brassica spp, the identity of the male determinant(s) remains elusive. Molecular analyses of the S locus have revealed the presence of a number of genes in addition to SRK and SLG. One, SLA (for S locus anther), although expressed exclusively in male reproductive tissues (Boyes and Nasrallah, 1995), has been determined not to function in SI, because self-incompatible lines of Brassica have been shown to contain nonfunctional SLA alleles (Pastuglia et al., 1997). Another study identified two additional S locus genes,  $SLL_1$  and  $SLL_2$  (for S locus-linked genes 1 and 2), also active in anther tissue (Yu et al., 1996). However, it seems unlikely that either of them encodes the male determinant because both were found to be nearly identical in the S lines investigated.

The sporophytic control of pollen compatibility in *Brassica* spp points to the pollen coating or tryphine, which is principally derived from the sporophytic tapetal cells that line the loculus, being the likely location for *S* gene products (Dickinson and Lewis, 1973; Heslop-Harrison et al., 1974; Doughty et al., 1993). In the final stages of pollen development, the tapetal cells degrade and their contents are transferred to the surface of the grains. Confirmation that *S* specificity resides in the pollen coating has come from a recent pollination bioassay study that also provided evidence that the male determinant was likely to be a protein and to have a molecular mass of <10 kD (Stephenson et al., 1997).

In a previous study, we analyzed the pollen coat proteins (PCPs) from several *B. oleracea* incompatibility haplotypes and identified a highly basic 7-kD protein termed PCP7 (renamed here PCP-A1, for pollen coat protein, class A, 1; class A PCPs are defined by their conserved cysteine motif), which binds the stigmatic SLG (Doughty et al., 1993). Generally, PCP-A1 binds SLGs regardless of *S* haplotype, casting doubt over whether it is a candidate for the male determinant of SI.

In subsequent studies with self-compatible *B. napus*, proteins similar but not identical to PCP-A1 were found in the pollen coat that bound an SLG and an *S* locus-related glycoprotein (SLR1; Hiscock et al., 1995; Dickinson et al., 1998). SLR1 is related to the SLGs and shares the same temporal and spatial pattern of accumulation (Lalonde et al., 1989; Trick and Flavell, 1989; Trick, 1990; Umbach et al., 1990). Although SLR1 is not encoded by the *S* locus and therefore cannot be involved directly in SI, there is some evidence to suggest that it plays a role in pollen adhesion (Luu et al., 1997); in this respect, interaction with a PCP may be significant. Another PCP (PCP1) has also been identified in *B. oleracea* that although closely related to PCP-A1, lacks the ability to bind stigmatic *S* proteins (Stanchev et al., 1996). Thus, PCP-A1 appears to be one member of a family of similar PCPs that may play a multiplicity of roles in the pollination process.

Here, we report the cloning of *PCP-A1* from a *B. oleracea* var *alboglabra* line homozygous for the  $S_{25}$  incompatibility haplotype, and we demonstrate that it is not linked to the *S* locus. *PCP-A1* also was found to show an exclusively gametophytic mode of expression. Sequence comparisons reveal it to be related to a superfamily of small cysteine-rich antimicrobial proteins found in both animal and plant systems. In the light of these data, we discuss the putative role of PCP-A1 in the SI response, and based on evidence for a gametophytic origin of several PCPs, we propose an alternative theory to explain sporophytic control of SI in *Brassica* spp.

# RESULTS

# Purification and Protein Sequencing of PCP-A1

PCP-A1 was purified from the pollen coating of three *B. oleracea* var *alboglabra* incompatibility haplotypes ( $S_{25}$ ,  $S_{29}$ , and  $S_{63}$ ) by using a combination of gel filtration, reverse phase, and cation exchange chromatography. In each case, the reverse phase and cation exchange HPLC chromatograms were very similar, and little variation in retention time was noted for PCP-A1 between the *S* haplotypes used in this study (data not shown). These preliminary observations indicated that PCP-A1 was unlikely to be highly polymorphic between *S* haplotypes, although it is possible that the very basic nature of the protein (pl of ~10) may have masked relatively minor amino acid differences using these techniques.

The purified proteins were subjected to N-terminal sequence analysis and were found to be blocked in all three cases; thus, proteolytic digests were conducted, and the resulting peptides were repurified and sequenced. The results of the amino acid sequence data are summarized in Figure 1 and reveal a relatively low level of sequence polymorphism between them. Interestingly, sequence variation was found not only between *S* haplotypes but also within them.

Two copurifying proteins are present in the  $S_{29}$  and  $S_{63}$  *B. oleracea* var *alboglabra* lines, with a conservative substitution at residue 9 (glutamine/glutamic acid) and a nonconservative substitution at residue 45 (threonine/methionine), respectively. Substitution between methionine and threonine residues at position 45 also extends to the other sequences (methionine in  $S_{29}$  PCP-A1 and threonine in  $S_{29}$  PCP-A1).



**Figure 1.** Summary of Amino Acid Sequence Data for PCP-A1 Derived from *B. oleracea* var *alboglabra* Plants Homozygous for the  $S_{25'}$ ,  $S_{29'}$  and  $S_{63}$  Incompatibility Haplotypes.

The sequences are aligned to the sequence derived from the  $S_{25}$ *cPCP-A1* clones ( $cS_{25}$ ). Sequences were obtained by proteolytic digestion of purified PCP-A1 and concatenated where joins could be inferred from other peptide sequences. The asterisks and vertical lines mark positions of sequence variation; the bracketed glutamine residue (Q) was cyclicized, causing blockage of the N terminus, and was removed by digestion with pyroglutamate aminopeptidase before sequencing; the dashes represent unassignable residues. The single residues placed below the sequences (E in  $S_{29}$ , M in  $S_{63}$ ) represent additional peptide sequences from those haplotypes. Digests and peptides produced are as follows: S25, a-chymotrypsin (SQEP-DKTCEVN, KKIL, TSCIKENNGNMY, and CRCQYPCP); S<sub>25</sub>, V8 protease (NNGNMYCRCQY); S25, pyroglutamate aminopeptidase treatment of N-terminally blocked chymotryptic peptide (KRKP-CYSQEPDKTCEVN);  $S_{29}$ ,  $\alpha$ -chymotrypsin (SQEPDKTCEV, TSCIKE-NNGNTY, and CRCQYPCP); S29, trypsin (KPCYSQEPDK and KPCYSEEPDK, TCEVNR, ATCVK, ENNGNTYCR, and CQYP); S<sub>63</sub>, α-chymotrypsin (TSCIGENNGNT/MY [7/3 ratio T/M] and CRC-QYPC); S<sub>63</sub>, trypsin (KP-YSQEPDKT-EVN; sample was not reduced and alkylated).

The most notable variation occurs in the  $S_{63}$ -derived sequence, with a highly nonconservative substitution at residue 39 from a very polar basic lysine residue to the small nonpolar glycine residue. The unassignable residues present in the unreduced  $S_{63}$  tryptic peptide are likely to be cysteines by inference from the other sequences obtained. No evidence was obtained from the  $S_{25}$  line to confirm the presence of copurifying PCP-A1 variants similar to those found in the other *S* lines studied; however, because the polypeptide sequences are incomplete, we cannot state unequivocally that other variants are not present in these samples. An amino acid compositional analysis was performed on the  $S_{25}$  PCP-A1 polypeptide, the results of which agree well with the predicted composition based on the *CPCP-A1* sequence, taking into account minor contamination (data not shown).

# Polymerase Chain Reaction–Based Cloning and Sequence Analysis of *PCP-A1*

The 5' and 3' rapid amplification of cDNA ends (RACE) cloning strategy employed was successful in obtaining clones that were in agreement with the protein sequence data for PCP-A1 from the  $S_{25}$  haplotype, as shown in Figure 2. The sequences obtained from the 3' RACE experiments (218 bp) were found to lack the oligo(dT) primer, which was lost during the cloning procedure. Clones obtained by 5' RACE all terminated within 15 bp of one another, the longest being 178 bp. Assembly of these overlapping clones produced a consensus sequence of 351 bp (*cPCP-A1*), which probably represents the majority of the *PCP-A1* transcript (see below), not including the poly(A) tail (Figure 2). The longest reading frame present in *cPCP-A1* utilizes the first ATG in the sequence and encodes a protein of 81 amino acids. A polyadenylation signal consensus sequence (AATAAA) is

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121	 tt	tat	tat	-+- ata	atc	att	+ cca	aaa	tag	att	+	ictt	gat	-+- gat	gta	tgt	+ att	 tat	ata	+ ccta
181	 ta	taa	att	-+- gaa	tgt	cta	-→+ att	ata	 taa	tat	+	gtt	taa	-+- tga	ttt	tto	+ aag	aat	aat	+ aaat
241	ga	ttt	aga	-+- aag	aag	aac	+ ttt	aaa	cat	ata	+ gtt	ctt	ttt	-+- tta	taa	gta	+ tgc	aga	aaa	+ ctct
301	 ct	aaa	aat	-+- atg	ttt	gtt	+ tta	caa	aaa	aaa	+	tca	gtt	-+- ttt	att	tta	+ aat	atg	aaa	+ ctaa
361	 ag	 tta	 cgt	-+- ttt	aat	tat	+ tat	aat	aac	G agG	E +	T AAC	<b>V</b> GGT	<b>1</b> -+- GAT	A TGC	Q TCA	K + GAA	R AAG	к  ААА.	P + ACCA
421	c	Y	s	Q -+	Е	P	D	ĸ	т	с	Е +	v	N	R	с	к	A	N	с	v
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541	Y	с	R	с -+	Q	Y	P +	c	P	P	+			-+-			+			+
	TA	ΓTG	CCG	ATG	rcaj	ATA	ICC,	TTG	200	TCC	Cta	att.	ttt	ttt	atc	tac	acc	aaa	act	caaa
601	tai	ata	ttt	ataa	ata	atad	cta	tga	ctt	tat	+ caa	aga	aat	-+- g <u>aa</u>	 taa	 <u>a</u> ac	a			

Figure 2. Nucleotide Sequence and Deduced Amino Acid Sequence of *PCP-A1*.

Noncoding regions of the gene are in lowercase, and coding regions are in uppercase. Lowercase boldface letters indicate intron splice donor/acceptor sequences and the stop codon. The putative polyadenylation signal sequence is underlined. The plus signs mark every tenth nucleotide. The deduced amino acid sequence is shown above the corresponding DNA sequence, and the putative signal peptide is denoted with boldface italics. The EMBL accession numbers for the cDNA and genomic polymerase chain reaction-derived clones are AJ005280 and AJ005281, respectively. present also at position 640, close to the end of the *cPCP-A1* 3' untranslated region. As mentioned above, comparison of the deduced amino acid sequence with those obtained by direct sequencing of the  $S_{25}$  PCP-A1 protein and the data from the amino acid composition analysis suggest that the *cPCP-A1* clone is authentic.

Further analysis of the deduced protein sequence identified a putative 26-amino acid hydrophobic signal peptide (Figure 2), which conforms to the von Heijne -3, -1 rule for signal cleavage sites (von Heijne, 1986), followed by a predominantly hydrophilic polypeptide. The position of the cleavage site is also in agreement with the predicted N terminus of the mature  $S_{25}$  PCP-A1 protein, as was determined by pyroglutamate aminopeptidase treatment and subsequent sequencing of the N-terminally blocked PCP-A1 peptide (see above). The mature form of the  $S_{25}$  PCP-A1 is estimated to have a relative mass of 6426 D and a pl of 9.26, values that are roughly in agreement with electrophoretic data obtained previously (Doughty et al., 1993).

To obtain data on the genomic structure of *PCP-A1*, we used primers designed to the extreme 5' and 3' ends of the coding region to amplify the sequence from *B. oleracea*  $S_{25}$  genomic DNA. A 550-bp fragment was amplified, and subsequent analysis of the sequence revealed 100% homology to *cPCP-A1*, except for the presence of a single 297-bp intron located close to the 3' end of the signal peptide coding region (Figure 2). The intron splice donor and acceptor sites GT/AG are in broad agreement with the consensus for plants (Brown, 1986), and the sequence is typically AT rich (82%) relative to the coding region (62%; Simpson et al., 1993).

Comparison of the full consensus sequence (aPCP-A1) to PCP1, a previously cloned member of the PCP-A class of PCPs (Stanchev et al., 1996), revealed some remarkable homologies within the noncoding regions of the genes (Figure 3). The percentage of nucleotide similarity between the coding regions is 60% (only 54% over the coding region of the mature PCP-A1), whereas the introns and 3' untranslated regions are 78 and 84% similar, respectively. The position of the intron also is perfectly conserved between gPCP-A1 and PCP1, splitting the codon for glycine at residue 21 in the signal peptide. Both introns are of a similar size, with that of gPCP-A1 containing an additional 13 bp, which appear to have been inserted as two small blocks rather than having been dispersed throughout the sequence. The position of the first polyadenylation signal sequence also is closely conserved within the context of the surrounding sequence homology.

Assuming that the overall structural homology of *PCP-A1* to *PCP1* extends both 5' and 3' of the cloned sequence, it appears that the *cPCP-A1* 5' untranslated region is full length (assuming that transcription start sites are usually 20 to 32 nucleotides from the TATA sequence), but the extreme 3' end of the transcript may be truncated by 20 to 30 nucleotides. Wider DNA homology searches failed to identify other closely related sequences in the databases; however,

the first 14 nucleotides of the intron were found to be identical to those at the extreme 5' end of the intron in the pollenspecific *BP4C* gene from *B. napus* (Albani et al., 1990). As for both *PCP-A1* and *PCP1*, the *BP4C* intron is located in the signal peptide coding region of the gene.

# PCP-A1 Transcripts Accumulate Specifically in Maturing Pollen Grains

To determine the temporal and spatial expression pattern of *PCP-A1*, we prepared RNA gel blots with mRNA isolated from a range of anther sizes covering the whole of pollen development as well as from pollen from freshly dehisced anthers, stigmas, and leaf tissue. A 165-bp probe representing the coding region of the mature PCP-A1 protein was used to

PCP-A1	TTTAAAACACAATCATTTCATTAGCTAAAATGAAAAACACGGTTAAACTT '	51					
PCP1	TTTAAAATATCPTTATTTCACTATCGAAGATGAAAGTTGCTTTCAAACCT	268					
PCP-A1	TCOCTTATTGOTTTTGTCATOCTCACAGTTCTTCTATTAGTTTGTATTT	101					
PCP1	TGGCTTATTGGITTTTTTATGCTCGCAATTTTTCTGTTTGTTTGTATTT	318					
PCP-A1	ACATTACAACTTCATGAATTATTATATAATCATTCCAAAATAGATTTT	151					
PCP1	TCATTTATAATTTTATTAATATATATATAATCATTCFAAAATAAAT	368					
PCP-A1	ACTTGATGATGTATGTATTTATATACCTATATAAATTGAATGTCTAATTA	201					
PCP1	ATTTGATGGCTTTTAT. TTTATATACCTATATTAATTGTATATCTAATTC	417					
PCP-A1	TATAATATTGTGTTTTAATGATTTT. TCAAGAATAATAAATGATTTAGAAA	250					
PCP1	TATAATATTGAGTTTAATGATTTIGTTAAGAGTAATCAATGATTAAGAAC						
PCP-A1	GARGAACTTTAAACATATAGTTCTTTTTTTATAAGTATGCAGAAAACTCT	300					
PCP1	GTAGATGTTTTAGACT TTCAGTTTTTCTGTTTATGAAGGAAATTCT	513					
PCP-A1		350					
PCP1	CTAAACATATGTTTTATAAATAATGATTTTTATTTTTAAAT	554					
PCP-A1	ATGAAACTAAAGTTAAGTTTTAATTAITATAATAACAGAGAAACGGTGA	400					
PCP1	CTARAACTAAAATTATGTTTTAATTATTATAATAACAGGTGAAACGGCGA	604					
PCP-A1	TTGCTCAGAAAAGAAAACCATGTTATTCACAAGAG	435					
PCP1	TAGCTCAGGAAAAAAAACTGACAACGAATGTAATCTTCCATTAATAAAC	654					
PCP-A1	CCAGACAAACATGTGAAGTCAATCGCTGCAAAGCCAACTGTGTCAAAAA	485					
PCP1	CAARGIGGAACATGTGCTGCCAACCAGTGTCAAGCCGCATGTGTCAAAAG	704					
PCP-A1	ACATAAGAAAATACTGGCTTTTAGCTGGTGCATTAAAGAAAATAACGGAA	535					
PCP1	GCATAAAGATGGGGTAGGTAAATGCACAACTAATCCAGACAAAA	748					
PCP-A1	ATATGTATTGCCGATGTCAATATCCTIGCCTCCCTAATTTTTTTTATCT	585					
PCP1	AAATGGGATGGATATGTTTTTTTTTTTTTTTGCCCTCGTGATTCTTTATCT	796					
PCP-A1	ACACCAAAACTCAAATAATAATATTATAATAATACTATGACTTTATCAAAGA	635					
PCP1	ACA. CAAAATTCAAATAA TATTATATATATATATATATATAT	845					
PCP-A1	аатдаатаааа 646						
PCP1	AAAGAATAAAA 856						

Figure 3. Gene Sequence Comparison of PCP-A1 and PCP1.

Positions of perfect homology are represented by vertical lines, periods represent gaps introduced to optimize alignment, and horizontal bars indicate the translation start codons. The intron and 3' untranslated regions of *PCP-A1* and *PCP1* are enclosed within boxes.



Figure 4. RNA Gel Blot Analysis of *PCP-A1* Expression in *B. olera*cea var alboglabra.

mRNA gel blot hybridized with a 165-bp *PCP-A1* coding region probe. Tissues from which the mRNA was extracted are indicated above the lanes. Stigmas were from newly opened flowers, leaves were fully expanded, and pollen was from freshly dehisced anthers. In the case of the anther stages, the size ranges refer to the flower bud sizes from which they were dissected, except for <2 mm for which whole buds were used. The arrow indicates the size of the transcript in base pairs.

avoid cross-hybridization with *PCP-A1*-related sequences (Stanchev et al., 1996). As shown in Figure 4, transcripts were detected in male reproductive tissues only, with expression initiating in 5- to 7-mm-long buds and reaching a maximum in 9- to 11-mm buds, with little or no expression after this stage. In a separate analysis, maximal expression was observed to occur in slightly smaller buds (data not shown). This variation between batches of buds collected on different occasions is most likely to have resulted from fluctuations in plant growth conditions in the glasshouse. The transcript size was estimated to be between 450 and 480 bp, which is consistent with the *cPCP-A1* protein.

A study of anther development in this Brassica line by transmission electron microscopy (J. Doughty and H.G. Dickinson, unpublished data) indicates that pollen is nearing full maturity when PCP-A1 expression is maximal. In 5- to 7-mm-long buds, the microspores are predominantly bicellular and the sporophytic tapetal cells that line the anther locule are just starting to degrade, although in some anthers they are fully intact. By the 9- to 11-mm stage, however, tapetal degradation is virtually complete and all grains are tricellular. Classically, components of the pollen coat, or tryphine, in Brassica spp have been considered to be derived from the tapetum; however, the "late" expression pattern displayed by PCP-A1, when tapetal dissolution is in an advanced state, inferred that PCP-A1 transcripts may accumulate in the microspores. To determine the precise site of PCP-A1 expression, we performed an in situ analysis with anthers isolated from buds in the 5- to 11-mm size range. The results of the analysis, as shown in Figure 5, confirmed that PCP-A1 demonstrates a purely gametophytic mode of expression. At the 6-mm bud stage, the tapetum is clearly still intact, but no signal was detected in this tissue, whereas transcripts were detected in the bicellular microspores, albeit at a low level (Figures 5A and 5C). In anthers from 8-mmlong buds, the tapetum is degrading (or occasionally degraded) with no signal being detectable, whereas high levels of *PCP-A1* expression are evident in the microspores that are tricellular by this stage (Figures 5B and 5D). By the 10-mm bud stage, tapetal degradation is complete and *PCP-A1* expression is maximal (Figure 5E). The failure of signal to be detected in some of the pollen shown in Figure 5 results from the restricted access of reagents to grains that have been incompletely sectioned due to the robust nature of the exine. Signal was not detected in any other anther tissue regardless of the stage of development or the use of a "sense" riboprobe as a control (Figure 5F).

### PCP-A1 Is Unlinked to the S Locus

To determine whether *PCP-A1* plays a role in conferring *S* specificity to pollen, we performed a mapping study using a highly polymorphic population of *B. napus* lines, as described by Parkin et al. (1995). The results of the analysis revealed *PCP-A1* to be situated just below the locus pN23b at the end of linkage group N9. N9 is an A genome linkage group (derived from *B. rapa*) and is homoeologous to portions of the C genome linkage groups N18 and N19. The *S* loci of *B. napus* map to distinct regions of the genome close to the pW150 loci on linkage groups N7 (A genome) and N16 (C genome) (Parkin, 1995).

Further, DNA gel blot analysis, as shown in Figure 6, indicated that *PCP-A1* has a variable copy number between different *Brassica S* lines, with a single or two closely linked copies present in the  $S_{25}$  *B. oleracea* var *alboglabra* line and two or three copies in the  $S_{29}$  and  $S_{63}$  lines (note that in Figure 6, the loading in lanes containing  $S_{29}$  DNA is slightly lower). The data from lanes containing the *B. napus* Westar DNA suggest that *PCP-A1* is present as a single-copy gene in each of the progenitor genomes—an observation in agreement with that obtained from the mapping study. Hybridization of the probe to filters of Arabidopsis genomic DNA failed to detect *PCP-A1* homologs in this species.

# PCP-A1 Is a Member of a Large Family of Structurally Related Cysteine-Rich Proteins That Includes the Plant Defensins

Analysis of the mature PCP-A1 polypeptide sequence reveals a backbone of cysteine residues, a feature that is shared by a superfamily of small, usually antimicrobial proteins found in both the plant and animal kingdoms. PCP-A1, along with PCP1, was found to be most similar to a class of cysteine-rich, polymorphic antimicrobial proteins termed plant defensins, which have a characteristic motif of eight cysteines. PCP-A1 also possesses eight cysteine residues,



Figure 5. In Situ Hybridization Study of *PCP-A1* Expression through Anther Development.

Eight-micron transverse sections hybridized with a digoxigenin-labeled 165-bp PCP-A1 coding region riboprobe are shown.

(A) and (C) Anther sections derived from a 6-mm-long flower bud hybridized with a *PCP-A1* antisense riboprobe. Pollen grains are binucleate; note the weak signal in pollen (blue color) and its absence in the tapetum. T and P represent tapetum and pollen, respectively.

(B) and (D) Anther sections from 8-mm-long flower buds hybridized with a *PCP-A1* antisense riboprobe. The pollen is trinucleate, and the tape-tum is degrading.

(E) Anther section from a 10-mm-long flower bud hybridized with a *PCP-A1* antisense riboprobe. The tapetum is fully degraded, and the *PCP-A1* hybridization signal in pollen is strongest from this anther stage.

(F) Anther section from a 10-mm-long flower bud hybridized with a PCP-A1 sense riboprobe as a negative control.

Bars in (A) to (F) = 15  $\mu$ m.

and as shown in Figure 7, their spacing is fairly well conserved to those of the plant defensins, although the intervening sequences are highly diverged. Like the plant defensins, PCP-A1 is hydrophilic in nature, is heat stable, and has a net positive charge (Doughty et al., 1993).

Structures have been determined for three plant defensins to date, Rs-AFP1 from *Raphanus sativus* (radish) seeds (Fant et al., 1994) and  $\gamma$ 1-P and  $\gamma$ 1-H from wheat and barley seeds (Bruix et al., 1993). In all cases, the structure comprises a triple-stranded antiparallel  $\beta$  sheet lying parallel to an  $\alpha$  helix. The Cys-X-X-Cys segment of the  $\alpha$  helix is connected by two disulfide bridges to the Cys-X-Cys segment of the third  $\beta$  strand, a structural motif known as the cystine-stabilized  $\alpha$  helix (Broekaert et al., 1995). The cystine-stabilized  $\alpha$  helix sequence motif is also present in PCP-A1; taken together with the fact that the positioning of the remaining four cysteines is similar to the plant defensions, it seems likely that they share the same three-dimensional structure.

To investigate this possibility further, purified  $S_{25}$  PCP-A1 was tested for the presence of free sulfhydryl residues by alkylation and radiolabeling with tritiated iodoacetamide. The results from this analysis confirmed that all of the cysteine residues form intramolecular disulfide bridges (data not shown), as is the case for defensins. In addition, a structural prediction was determined for PCP-A1 by computer homology modeling using the MODELLER package of Sali and Blundell, (1993), based on the structure determined for  $\gamma$ 1-P from wheat endosperm. The model that was obtained



**Figure 6.** Gel Blot Analysis of Genomic DNA from Self-Incompatible *B. oleracea* var *alboglabra* (Lines  $S_{25^{r}} S_{29^{r}}$  and  $S_{63}$ ) and Self-Compatible *B. napus* (Westar).

The restriction enzymes used to digest the genomic DNA are indicated above the lanes in which they were used. Molecular length marker positions are indicated at left in kilobases. The blot was hybridized with a 165-bp *PCP-A1* probe representing the coding region of the mature protein only. West. represents lanes containing Westar DNA. using this procedure, as shown in Figure 8, closely resembles that determined by nuclear magnetic resonance spectroscopy for  $\gamma$ 1-P,  $\gamma$ 1-H, and Rs-AFP1; thus, we predict that PCP-A1 also possesses this structure.

Although PCP-A1 is clearly related to this group of proteins, all other consensus residues found among the plant defensins are not present in PCP-A1. These include two glycines (positions 15 and 37 relative to the start of PCP-A1 in Figure 7), a glutamic acid (position 31), an aromatic residue (tryptophan or phenylalanine at position 13), and frequently, a serine at position 10. Similarly, the PCP-A1-like proteins PCP1 and Bp4C are significantly diverged from the defensin group, although PCP1 does retain the conserved glycine residues (Figure 7).

# The *S*<sub>29</sub> SLG Exists as Monomers and Homodimers in Vitro, Both of Which Form Complexes with PCP-A1

In a previous study, we reported that PCP-A1 binds the SLG in an isoelectric focusing band-shift assay (Doughty et al., 1993). We investigated this interaction further by using a ligand blotting procedure in which stigmatic proteins are first separated by nonreducing gel electrophoresis and then transferred to membrane and "probed" using iodinated PCP-A1. A polyclonal antibody specific for the  $S_{29}$  SLG (SLG<sub>29</sub> var-B3) was also used in this study to identify unambiguously the position of SLG on the blots.

Protein gel blots of reduced and nonreduced crude stigmatic protein extracts derived from plants homozygous for the  $S_{29}$  haplotype revealed the SLG to be present in both monomeric and dimeric forms, as shown in Figure 9A. Under reducing conditions, the  $S_{29}$  SLG monomer migrates as 3 or 4 bands representing different glycoforms with molecular masses of  $\sim$ 53 to 55 kD as is typical for SLGs (Umbach et al., 1990) (Figure 9A, lane 2); however, under nonreducing conditions, an additional abundant ~100-kD band is present that cross-reacts with the anti-S29 SLG antibody (Figure 9A, lane 3). The intensity of this upper band was always found to be greater than that representing the SLG monomer, suggesting that the dimeric form may be the preferred structure for the S29 SLG. Very weak SLG29 var-B3 cross-reacting bands also were detected with molecular masses >100 kD on some blots, which may represent higher SLG multimers or nonspecific cross-reaction of the antibody to other proteins in the sample.

Low-level artifactual formation of SLG multimers by oxidative cross-linking during tissue extraction has been reported in the literature (Stein et al., 1996); therefore, their presence cannot be ruled out here. However, such crosslinking was found to be irreversible by heating in reducing sample buffer and therefore cannot account for the reversible SLG dimerization reported here. Because SLG readily forms homodimers, it is conceivable that SLG also may associate with the receptor domain of the SRK (with which it is 83% identical at the amino acid level in the  $S_{29}$  haplotype),

PCP-A1	QKRKP	QY SQEPDKTK	JEVN-ROKANC	VKKHKKI-LAFTS	ĞI KENNGNMYĞRĞQY PĞP P
PCP1	QEKKTDNQ	CULPLINKS	AAN-QQQAAC	VKRHKDG-V <mark>G</mark> K	drinpdkkmrdidfylcpr
Bp4c	QKKN	CPRKIPIKGSYC	APT-ICLDKC	KK-QHGT-VGS	CAEEKGFCNCACK
Rs-AFP1	QKL¢	CERPSCIMSCVC	GNINNACKNOC	INLEKAR-HGS	CNYVFPA-HKCICYFPC
At-AFP1	QKL	CERPSCTWSCVC	GNSNACKNOC	INLEKAR-HGS	CNYVFPA-HKCICYFPC
Hs-AFP1	DGVKL	CDVPSGTWSCHC	<u>ssssk</u> dsog	KDREHFAYCC - A	CHYQFPS-VKCFCKRQC
Ct-AMP1	NLC	GERASLTWIGNO	GNTGHODTOC	RNWESAK-HGA	CHKR-GN-WKCFCYFDC
sia2	RV	CMKGSAGFKGLC	MRDQNdaQVC	LQ-EGWG-GG <b></b> N	dpgvmrddkdirdd
γ1-P	KI	GRRRSAGEKGPC	MSNKNCAQVC	¦QQ-EGWG-GG <b>-</b> -N	dpgpfrrdrdir.d
PPT	RT	desogshrafnata	vresndasvc	QT-EGFI-GGN	GRAFRRRGFGTRNG
J1	кі	GEALSGNFKGLO	LSSRDØGNV	RR-EGET-DGS	digfrlqdfdtkpda
FST	RE	GKTESNTEPGIG	TTKPP CRKAC	IS-EKFT-DGH	gskllrrdidtkpd
		₹ 1		·	<u>†                                    </u>
		1			-

Figure 7. Amino Acid Sequence Alignment of PCP-A1 with Similar Pollen-Borne Proteins and Various Plant Defensins.

The upper group, in addition to PCP-A1, includes pollen-specific polypeptides PCP1 (Stanchev et al., 1996) and Bp4C (Albani et al., 1990) from *B. oleracea* and *B. napus*, respectively. The plant defensins shown (lower group) are Rs-AFP1 from *R. sativus* seeds (Terras et al., 1992), At-AFP1 from *Arabidopsis thaliana* seeds (Terras et al., 1993), Hs-AFP1 and Ct-AMP1 from *Heuchera sanguinea* and *Clitoria ternatea* seeds, respectively (Osborn et al., 1995), Si $\alpha$ 2 from *Sorghum bicolor* seeds (Nitti et al., 1995),  $\gamma$ 1-P from *Triticum aestivum* seeds (Colilla et al., 1990), PPT from *Petunia inflata* styles (Karunanandaa et al., 1994), J1 from *Capsicum annuum* fruit (Meyer et al., 1996), and FST from tobacco flowers (Gu et al., 1992). The predicted secondary structure of PCP-A1 is indicated above the sequence with horizontal bars and a cylinder representing the  $\beta$  strands and an  $\alpha$  helix, respectively. Linked arrows indicate pairs of cysteine residues involved in disulfide bridge formation as determined for a number of defensins (Fant et al., 1994, for Rs-AFP1; Bruix et al., 1993, for  $\gamma$ 1-P). Hyphens represent gaps introduced to optimize the alignment. Boxed amino acids indicate the eight conserved cysteine residues and those residues that are generally conserved among plant defensins. Signal peptides have been omitted from the alignment as has a C-terminal propeptide domain from FST.

forming a heterodimer that could explain the high molecular weight cross-reacting proteins in the sample.

When blots of nonreduced crude stigmatic protein extracts were incubated with <sup>125</sup>I-labeled PCP-A1, signal was associated with both the monomeric and dimeric forms of SLG (Figure 9B). In addition, some binding to higher molecular weight species with similar mobilities to those weakly detected by the anti-SLG antibody was detected also. Samples that were reduced by heating in sample buffer containing β-mercaptoethanol before blotting failed to bind PCP-A1 (data not shown). As shown in Figure 9C (lanes 1 and 2), separation of the crude extract into soluble and microsomal fractions permitted the resolution of a previously unidentified protein with a molecular mass of  $\sim$ 48 kD that also bound the labeled PCP-A1. This protein failed to cross-react with the SLG<sub>29</sub> var-B3 antibody (Figure 9C, lanes 3 and 4) and was present in both soluble and microsomal fractions, appearing to be enriched in the latter.

Results from isoelectric focusing band-shift assays reported previously (Doughty et al., 1993) found no evidence to suggest that PCP-A1 bound stigmatic proteins other than SLG, although stigmatic extracts were not enriched for the microsomal fractions, as is the case here. In addition, electrophoresis of protein complexes may cause dissociation of weak protein–protein interactions, which would be preserved with the ligand blotting procedure used in this study. Interestingly, a significant proportion of the SLG pool also was detected in the microsomal fraction, which probably represents those SLG molecules still present in the secretory pathway at the time of extraction, an observation also noted by Stein et al. (1996). PCP-A1 binding to two diffuse bands with molecular masses >100 kD also were slightly enriched in the microsomal fraction; whether either of these represents binding to SRK remains to be determined.

# DISCUSSION

Investigations on the molecular basis of the *Brassica* SI system in recent years have cast some light on the recognition molecules and mechanisms operating in stigmatic tissues that ultimately lead to the rejection of self-pollen. In contrast, comparatively little is known of the pollen-borne components of the *Brassica* SI system. At the most simplistic level, each *S* haplotype needs only to encode one pollen-borne *S* specific ligand for SI to function. Similarly, a single *S* factor would be sufficient for SI to function in stigmatic tissue; however, the fact that both the stigmatic SLG and SRK are essential components hints at a higher level of molecular complexity in terms of "activation" of the SI system. In this study, we have described the characterization of PCP-A1, a small pollen-borne protein that binds SLGs, and importantly, we have demonstrated that it is not encoded by the *S* locus.

It is now emerging that PCP-A1 belongs to a family of PCP-A1–like proteins present in the pollen coats of *Brassica* 

spp. A related PCP-A class protein, PCP1, previously identified through its copurification with PCP-A1, appears not to bind SLGs (Stanchev et al., 1996). However, data from studies on *B. napus* pollen coating have revealed that a number of PCP-A1-like proteins are present with distinct binding specificities (Hiscock et al., 1995; Dickinson et al., 1998; J. Doughty, unpublished results). One such PCP has been demonstrated to bind specifically to the stigma-specific SLR1 protein, whereas others bind only an SLG. SLR1, although similar to SLGs, does not function in SI but is believed to play a role in pollen adhesion (Luu et al., 1997). This finding highlights the importance of different members of the PCP-A protein family in the pollination process; indeed, recent findings indicate that the male determinant of SI in B. oleracea is located in the pollen coating and has size and charge characteristics broadly similar to that of PCP-A1 (Stephenson et al., 1997); therefore, it is conceivable that a member of this family may be responsible for determining S specificity.

Comparison of the *PCP-A1* and *PCP1* gene sequences revealed an unusual pattern of sequence homology, indicating that gene conversion is likely to have contributed to the evolution of the *PCP-A* gene family. Data on the genomic organization of *PCP1*-like genes previously revealed that gene copy numbers vary between different *B. oleracea* lines and also indicated that the sequences were clustered in the genome, a situation that would facilitate gene conversion events. *PCP-A1* copy number, also found to be variable between *Brassica* lines, was estimated at one to three copies per haploid genome, and evidence from the protein se-



Figure 8. Homology Model of PCP-A1.

The model is based on the three-dimensional structure of  $\gamma$ 1-P, which was determined by nuclear magnetic resonance spectroscopy (Bruix et al., 1993).  $\beta$  strands are represented by broad arrows, and the  $\alpha$  helix is indicated by a helical ribbon. The four disulfide bridges are shown as yellow and black balls and sticks. quence data indicates that at least two *PCP-A1* alleles are expressed in two of the lines studied.

One of the key features of the PCP-A1 protein sequence is the presence of a structurally important motif consisting of eight cysteine residues. This motif is shared with the plant defensin family of proteins. Based on sequence homologies, comparative molecular modeling, and the lack of free sulfhydryl residues in PCP-A1, we predict that PCP-A1 also adopts the same robust structure as defensins, thus explaining its inherent stability. Some plant defensins, such as Rs-AFP2, exhibit antifungal activity with characteristic specificities (Terras et al., 1992, 1993), whereas others such as Si $\alpha$ 2 lack antifungal activity but act as  $\alpha$  amylase inhibitors (Bloch and Richardson, 1991). PCP-A1 and the plant defensins belong to a wider family of cysteine-rich proteins also found in vertebrates and invertebrates, many of which are thought to play roles in defense against microbes (reviewed in Hoffmann and Hétru, 1992; Lehrer et al., 1993). One such protein, drosomycin, isolated from the fruit fly (Drosophila), has been demonstrated to be structurally related to plant defensins (Landon et al., 1997). This family of proteins thus appears to have maintained a structure that is highly stable and suited to an extracellular environment but yet has evolved to interact with a wide range of different target molecules-from enzymes to as yet unidentified receptors on fungal hyphae. These characteristics would be well suited to molecules involved in intercellular signaling during pollination.

The finding that *PCP-A1* is unlinked to the *S* locus clearly rules out a role for it conferring S specificity to pollen. However, our data does not discount the possibility that PCP-A1 could function as a cofactor in activation of the S receptor complex, in conjunction with the as yet unidentified male determinant of SI, to elicit rejection of self-pollen. A similar scenario has been reported in Papaver rhoeas in which a pollen-specific membrane-bound glycoprotein has been identified (SBP; for S protein binding protein) that binds stigmatic S proteins regardless of S genotype (Hearn et al., 1996). The authors envisage SBP acting as an accessory receptor or coreceptor whose binding to stigmatic S proteins is necessary for their interaction with an S-specific pollen receptor. Data from the ligand blotting experiments reported here verified PCP-A1's high affinity for the SLG and also revealed that SLG readily forms homodimers in vitro. Further, the SLG homodimer also binds PCP-A1, implying that the binding site is not blocked by dimerization. Although it is impossible to state with certainty, it seems reasonable to assume that SLG dimerization also occurs in vivo and that upon pollination at least two PCP-A1 molecules bind the homodimer.

If indeed PCP-A1 does act as a cofactor in activation of the *S* receptor complex, its binding to SLG may be a prerequisite for SLG binding of the pollen *S* factor. The complex thus formed, which could involve SLG in dimeric form, may then bring about activation of SRK receptors at the plasma membrane either by causing their dimerization, as is typical for



**Figure 9.** Immunodetection of Monomeric and Dimeric Forms of  $SLG_{29}$  and Identification of Protein–Protein Interactions between <sup>125</sup>I-Labeled PCP-A1 and Stigmatic Proteins by Ligand Blotting.

(A) Immunodetection of monomeric and dimeric forms of  $SLG_{29}$  after separation of stigmatic protein extracts by reducing and nonreducing SDS-PAGE. Lane 1 contains  $S_{29}$  stigmatic protein extract (reduced) blotted and stained with Coomassie Brilliant Blue R 250; lane 2, as given for lane 1, but probed with the  $SLG_{29}$  var-B3 polyclonal antiserum; and lane 3, as given for lane 2, except that proteins were separated and blotted under nonreducing conditions before immunodetection of  $SLG_{29}$ .

**(B)** Detection of PCP-A1 binding proteins in crude stigmatic protein extracts by ligand blotting. Lane 1, autoradiography of an  $S_{29}$  stigmatic protein gel blot (nonreducing) probed with <sup>125</sup>I-labeled PCP-A1; and lane 2, protein blot as given for lane 1, except that it was probed with the SLG<sub>29</sub> var-B3 polyclonal antiserum.

**(C)** Detection of PCP-A1 binding proteins in stigmatic soluble and microsomal membrane protein fractions. Lane 1 contains the soluble protein fraction derived from  $S_{29}$  stigmas probed with <sup>125</sup>I-labeled PCP-A1 (as given for **[B]**, lane 1); lane 2, microsomal membrane protein fraction (Triton X-114-soluble components of membranes) derived from  $S_{29}$  stigmas probed with <sup>125</sup>I-labeled PCP-A1; and lanes 3 and 4, same contents as given for lanes 1 and 2, respectively, except that lanes 3 and 4 were probed with the SLG<sub>29</sub> var-B3 polyclonal antiserum.

Immunoblots shown in (A) and (B) were developed using chromogenic substrates, whereas those in (C) were developed using chemiluminescent substrates. Arrows indicate monomeric and dimeric forms of SLG; in (C), the bottom arrow indicates the position of the microsomal membrane-associated PCP-A1 binding protein (~48 many single-pass transmembrane receptor kinases (Ullrich and Schlessinger, 1990), or by activating preexisting inactive SRK dimers. Alternatively, PCP-A1 may act to regulate and stabilize association between SLG and SRK, forming a linked heterodimer that would then be competent to bind the male determinant. The ligand blotting experiments also revealed the association of PCP-A1 with proteins enriched in microsomal membrane fractions, including two diffuse bands having molecular masses >100 kD as well as a distinct  $\sim$ 48-kD protein. The significance of these interactions cannot be determined until the identity of the membrane proteins has been established; however, it is tempting to speculate that PCP-A1 may bind SRK (mature molecular mass is  $\sim$ 116 kD), given the relatively high level of sequence homology between its receptor domain and SLG (83% amino acid identity).

In situ localization of *PCP-A1* transcripts revealed them to be specifically localized to pollen grains late in development, with maximal expression being reached at the trinucleate stage. This exclusively gametophytic mode of expression is somewhat surprising because the pollen coating previously had been considered to be derived principally from the tapetal cells that line the anther locule. Interestingly, *PCP1*, the protein product of which is also located in the pollen coating, was found to exhibit an identical temporal and spatial expression pattern to *PCP-A1* (Stanchev et al., 1996). We now believe that a number of coat proteins are the product of gametophytic expression coupled with subsequent protein export from the pollen protoplast into the loculus and accretion onto the exine surface.

To date, two alternative theories have been proposed to account for the sporophytic nature of SI in Brassica spp, in which pollen carries the products of the two S alleles of the parent plant. One suggests that the pollen-borne determinant of SI is expressed premeiotically (Pandey, 1970), whereas the other theory, perhaps more plausible in light of current research (Stephenson et al., 1997), predicts that the factor is tapetal synthesized and transferred to the pollen coat (Heslop-Harrison, 1968; Dickinson and Lewis, 1973; Heslop-Harrison et al., 1974). Based on the expression patterns exhibited by PCP-A1 and PCP1 described above, we propose an additional hypothesis by which gametophytic expression of Sgenes in pollen may result in a sporophytic-like S phenotype. For example, in a plant heterozygous for the  $S_1$  and  $S_2$ haplotypes, meiotic segregation ensures an equal population of  $S_1$  and  $S_2$  pollen in each anther. However, gametophytic expression of S genes and export of the protein products from the pollen into the thecal fluid of the loculus would allow mixing of the  $S_1$  and  $S_2$  determinants with other

kD) in lane 2. Molecular masses, calculated from relative positions of molecular mass markers, are indicated at left in kilodaltons.

molecules derived from the tapetum and pollen. In late stages of pollen maturity, the deposition of this mixture onto the surface of the grains would then result in a coating carrying both  $S_1$  and  $S_2$  male determinants.

Although the precise role of PCP-A1 has yet to be determined, its interaction with SLG further highlights the molecular complexity of the SI system in *Brassica* spp. In addition, PCP-A1 is one member of a family of PCPs that are emerging as playing an important part in pollen–stigma interactions.

# METHODS

### **Plant Material**

Brassica oleracea var alboglabra homozygous for the incompatibility haplotypes  $S_{25}$ ,  $S_{29}$ , and  $S_{63}$  (supplied by Horticultural Research International, Wellesbourne, Warwick, UK) and *B. napus* cv Westar were grown in a greenhouse with a 16-hr-day/8-hr-night photoperiod provided by supplementary lighting. A highly polymorphic population (N-fo-61-9) (Parkin et al., 1995) of doubled-haploid *B. napus* lines (derived from a cross between a resynthesized *B. napus* line SYN1 and a British winter cultivar N-o-9) was kindly made available by D.J. Lydiate (John Innes Centre, Norwich, UK) for mapping *PCP-A1*.

### **Purification of PCP-A1**

Pollen coat protein (PCP) extracts were prepared from 600 mg of pollen collected from freshly dehisced anthers and fractionated by gel filtration (Superdex 75 column; Pharmacia Biotech, Uppsala, Sweden), as described previously (Doughty et al., 1993). The PCP-A1-containing fraction was then separated further by reversed phase HPLC (Techogel C<sub>18</sub> silica, 5- $\mu$ m beads, 300 Å pore size, 2.1  $\times$  250 mm; HPLC Technology Ltd., Macclesfield, UK); proteins were eluted in a linear gradient of 15 to 45% (v/v) acetonitrile 0.1% (v/v) TFA over 60 min. Protein fractions were collected manually, dried in a rotary vacuum concentrator, resuspended in 50 mM potassium phosphate, pH 7.0, and screened for SLG binding activity (Doughty et al., 1993; Stephenson et al., 1997). PCP-A1 was purified to homogeneity by cation exhange HPLC (TSK-GEL, SP-NPR column, 2.5-µm nonporous beads, 4.6  $\times$  35 mm; TosoHaas, Montgomeryville, PA), and proteins were eluted in a linear gradient of 0 to 500 mM NaCl, 20 mM sodium acetate, pH 5.5, over 30 min.

# Isolation of mRNA and 5' and 3' Rapid Amplification of cDNA End Polymerase Chain Reaction Cloning of *PCP-A1*

Polyadenylated RNA was isolated from anthers (derived from *B. ole-racea* var *alboglabra*, homozygous for the  $S_{25}$  haplotype) covering all stages of pollen development by using the QuickPrep Micro mRNA purification kit (Pharmacia Biotech). One microgram of mRNA was subjected to first-strand cDNA synthesis by using an oligo(dT) primer incorporating an Smal restriction site (underlined) at its 5' end (5'-TGT<u>CCCGGGT</u><sub>18</sub>-3'), and amplification was conducted using this primer in conjunction with a PCP-A1 degenerate primer incorporating a BamHI restriction site at its 5' end (5'-GCGGATCCCAGGAGC-CIGATAA[G/A]ACITG[C/T]GA[A/G]GT-3', where I represents inosine)

based on the peptide sequence QEPDKTCEV. Polymerase chain reaction (PCR) was performed for 40 cycles at 94°C for 30 sec, 65°C for 1 min, and 72°C for 1 min followed by a final 5-min extension step at 72°C. Amplified products (250 to 270 bp) were gel purified (QIAEX II gel extraction kit; Qiagen Ltd., Dorking, UK) and cloned directly into the pGEM-T cloning vector (Promega Ltd., Southampton, UK), following the manufacturer's protocol.

Clones that represented putative *PCP-A1* transcripts were identified by a second PCR reaction by using the above PCP-A1 degenerate primer and a second degenerate primer (5'-TGT<u>CCCGGG</u>GTACAT-ITTICC[G/A]TT[G/A]TT[T/C]TC-3'; Smal restriction site underlined) based on the peptide sequence ENNGNMY. 5' Rapid amplification of cDNA ends (RACE) cloning of the *PCP-A1* cDNA was performed using a 5'/3' RACE kit (Boehringer Mannheim, Lewes, UK), following the manufacturer's instructions. First-strand cDNA was synthesized using a *PCP-A1* sequence–specific primer (5'-CGTTATTTTCT-TTAATGCACGAGG-3'), the sequence being obtained from the 3' RACE cloning detailed above, and amplification was performed with a *PCP-A1* nested primer 5'-GTTGGCTTTGCAGCGATTGAC-3' in conjunction with an anchor primer supplied with the kit. A PCR product of ~220 bp was obtained that was cloned using the pGEM-T cloning vector (Promega).

# **RNA Gel Blot Analysis**

Polyadenylated mRNA (450 ng) from leaves, stigmas, pollen, and anthers derived from a range of bud sizes (whole flower buds used for size  $\leq 2$  mm) were separated on 1.5% agarose–formaldehyde gels and blotted onto Hybond-N nylon membrane (Amersham Life Sciences Ltd., Little Chalfont, UK) by using standard procedures (Sambrook et al., 1989). After transfer, the blot was rinsed briefly in diethyl pyrocarbonate–treated water, and RNA was immobilized on the membrane by UV cross-linking (120,000  $\mu$ J cm<sup>-2</sup>) and baking for 1 hr at 80°C. Blots were prehybridized for 2 hr at 50°C (0.5 M sodium phosphate, pH 7.2, 7% SDS, 10 mg mL<sup>-1</sup> BSA). After the addition of the probe (see DNA gel blot analysis), blots were hybridized for 16 hr at 50°C. Washing was for 10 min at 50°C (2 × SSC [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS), followed by an additional 5 min wash at 50°C (1 × SSC and 0.1% SDS).

#### DNA Gel Blot Analysis and S Linkage Study

Genomic DNA was extracted from young leaves by using the cetyl triethylammonium bromide procedure (Draper et al., 1988). Ten micrograms of DNA was digested with the appropriate enzymes and separated on an 0.8% agarose gel and transferred to Hybond-N membrane and probed by using standard procedures (Sambrook et al., 1989). Labeling of the *PCP-A1* probe (165 bp—coding region of the protein minus the putative signal peptide) was conducted by random hexamer priming with the Klenow fragment of DNA polymerase I, using the Prime-a-Gene Labeling System (Promega), with modifications to the deoxynucleotidetriphosphates mix to allow for double labeling with dATP and dCTP ( $\alpha$ -<sup>32</sup>P, 100 µCi, 6000 Ci/mmol each). Mapping of *PCP-A1* was performed as described by Parkin et al. (1995).

# Genomic PCR

Genomic DNA (10 ng) from the  $S_{25}$  haplotype, isolated as described in the previous section, was used as a template. Primers designed to

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the extreme 5' (5'-CATTAGCTACCATGGAAAACACGG-3') and 3' (5'-GGGAGGGCAAGGATATTG-3') ends of the *PCP-A1* coding region were used in the following amplification protocol: 95°C for 1 min (95°C for 30 sec, 50°C for 1 min, and 72°C for 1 min) three times and (94°C for 30 sec, 53°C for 1 min, and 72°C for 1 min) 36 times, followed by a 5-min extension at 72°C. PCR products were cloned as described above in the section titled Isolation of mRNA and 5' and 3' Rapid Amplification of cDNA End Polymerase Chain Reaction Cloning of *PCP-A1*.

# **Protein Sequencing**

#### **N-Terminal Sequencing**

All sequencing reactions were run on an Applied Biosystems (ABI) 470A, 473A, or 494A Procise protein sequencer (Perkin-Elmer Ltd., Applied Biosystems Division, Warrington, UK). Liquid samples were applied to a glass fiber disc pretreated with polybrene to limit sample wash-out.

#### Proteolytic Digests

α-Chymotrypsin (sequencing grade; Boehringer Mannheim) digests were conducted in 100 mM Tris, pH 8.0 (1:10 enzyme/substrate ratio at 37°C for 6 hr). Endoproteinase Glu-C (V8 protease; sequencing grade from Promega) digests were performed in 100 mM ammonium bicarbonate, pH 8.2 (1:10 enzyme/substrate ratio, 37°C for 16 hr). Trypsin (modified trypsin; sequencing grade from Promega) digests were performed in 100 mM Tris, pH 8.0 (1:10 enzyme/substrate ratio; 37°C for 16 hr). Pyroglutamate aminopeptidase (sequencing grade; Boehringer Mannheim) digests were conducted in 100 mM sodium phosphate, pH 8.0, 10 mM disodium EDTA, 5 mM dithioerythritol, 5% glycerol (v/v; 1:20 enzyme/substrate ratio; initially at 4°C for 16 hr and then at 25°C for an additional 4 hr). Before digestion, samples were reduced, alkylated, and repurified using an Aquapore RP-300 column (100  $\times$  2 mm; Perkin-Elmer). Reduction and alkylation were achieved by dissolving the protein in 6 M guanidine hydrochloride, 0.5 M Tris, pH 8.0, and 2 mM EDTA. DTT was then added to a final concentration of 1 mg/mL, and the mixture was incubated at 37°C for 2 hr. 4-Vinylpyridine (1  $\mu$ L) was then added per 100  $\mu$ L of solution, and the mixture was incubated at 37°C for 1 hr. After proteolytic digestion, the cleavage products were repurified using an Aquapore OD-300 column (100  $\times$  2 mm; Perkin-Elmer) and N-terminally sequenced.

#### **DNA Sequencing**

Sequencing reactions were conducted using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequence analysis was performed on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA).

# **Antibody Production**

The SLG<sub>29</sub> var-B3 polyclonal antiserum was raised to a 12-amino acid, S<sub>29</sub>-specific synthetic peptide (YLFKDDFLVHRS). Rabbits were immu-

nized with 100  $\mu$ g of the peptide cross-linked to BSA (cross-linked with 1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide [Pierce and Warriner Ltd., Chester, UK]) and boosted three times with 50  $\mu$ g at 2-week intervals.

#### **Protein Gel Blotting**

All protein extracts were resolved on 12% SDS-polyacrylamide gels and blotted onto Immobilon-P (Millipore) membrane, according to Towbin et al. (1979), except that methanol was omitted from the buffer. Where protein samples were separated under nonreducing conditions, SDS and 2-β-mercaptoethanol were omitted from the standard sample buffer (0.125 M Tris-HCl, pH 6.8, 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-β-mercaptoethanol, and 0.01% [w/v] bromophenol blue), and 5 mM iodoacetamide was included for the ligand blotting experiments. Blots were blocked for 1 hr in TBS, 0.3% casein, and 0.1% Tween 20, and incubated with the SLG<sub>29</sub> var-B3 antiserum (1:250 dilution in blocker) overnight. Antigen-antibody complexes were detected using a 1:10,000 dilution (in blocker, 1 to 2 hr at 20°C) of a goat anti-rabbit IgG-alkaline phosphatase secondary antibody (Sigma). Blots were washed three times for 10 min in blocker and then equilibrated for 5 min in 5 mL of 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris-Cl, pH 9.5, before the addition of 16 µL of 5-bromo- 4-chloro-3-indolyl phosphate and 33 µL of nitro blue tetrazolium (Boehringer Mannheim). The color reaction was stopped by rinsing the blots in double-distilled H<sub>2</sub>O. For chemiluminescent detection, an enhanced chemiluminescence Western Blotting system was used (Amersham Life Sciences Ltd.), and the manufacturer's guidelines were followed.

# Ligand Blotting

Purified PCP-A1 protein (10 µg) was labeled with iodine-125 (~0.85 µCi µg<sup>-1</sup>), as described previously (Doughty et al., 1993). Blots were prepared as described under Protein Gel Blotting, blocked for 2 hr in 10 mM potassium phosphate, pH 7.0, 0.1% Tween 20 (Sigma), and then incubated overnight in 5 mL of blocker containing 0.25 µg of labeled PCP-A1. Blots were washed three times for 10 min each in blocker before exposing to film. All steps were conducted at room temperature.

#### Assay for Free Sulfhydryl Residues

Lyophilized PCP-A1 (0.25  $\mu$ g), purified as described above, was resuspended in 50  $\mu$ L of 6 M guanidine-HCl, 0.5 M Tris-HCl, pH 8.0. <sup>3</sup>H-Iodoacetamide (1  $\mu$ Ci) was added to the sample that was then incubated for 5 min at 37°C. Unlabeled iodoacetamide was added to a final concentration of 100 mM, and the sample incubated for a further 55 min at 37°C. A duplicate sample was treated identically except for the omission of <sup>3</sup>H-iodoacetamide from the buffer. Both samples were rechromatographed on an Atlantis C5 reverse phase column (150 × 1 mm; Phenomenex, Macclesfield, UK), and the peak corresponding to PCP-A1 was measured for incorporation of hydrogen-3. No incorporation of hydrogen-3 was detected, and the retention time of PCP-A1 was identical to that of the control sample.

# Preparation of Stigmatic Protein Extracts and Microsomal Fractions

All extraction procedures were conducted in the presence of protease inhibitors (leupeptin [10 µg mL<sup>-1</sup>], phenylmethylsulfonyl fluoride [1 mM], pepstatin-A [1  $\mu$ g mL<sup>-1</sup>], and aprotinin [10  $\mu$ g mL<sup>-1</sup>]) on ice, unless stated otherwise, and all samples were stored at -80°C. Crude stigmatic protein extracts were obtained by homogenizing 50 stigmas in 50 µL of extraction buffer (50 mM potassium phosphate, pH 7.0, and 1% Triton X-114). The homogenate was then centrifuged at 4000g for 10 min to remove cellular debris. Stigmatic microsomal fractions were prepared by homogenizing 200 stigmas in 200 µL of 50 mM potassium phosphate buffer, pH 7.0. The homogenate was then centrifuged twice at 4000g for 10 min to remove cellular debris, and the supernatant was recentrifuged at 100,000g at 2°C for 30 min to pellet the microsomal membrane fraction. The supernatant was retained and represented the soluble protein fraction. The pelleted microsomes were then washed in 200 µL of extraction buffer, repelleted, solubilized in 100 µL of extraction buffer, including 1% Triton X-114 (30 min on ice), and centrifuged for another 30 min at 100,000g. The resulting supernatant contained solubilized membrane proteins.

### **PCP-A1 Structural Prediction**

The structure for PCP-A1 was predicted by homology modeling using the MODELLER package of Sali and Blundell, (1993). The PCP-A1 sequence was aligned to that of the plant defensin  $\gamma$ 1-P from *Triticum aestivum*, for which the three-dimensional structure has been established by NMR spectroscopy (Bruix et al., 1993). The conserved cysteines were used as the basis for the alignment.

### In Situ Hybridization

Excised anthers were cut transversely and fixed in freshly prepared 4% paraformaldehyde for 16 hr at 4°C. Tissues were then embedded in Paraplast Xtra (Sigma), sectioned (7 to 10 µm), and prepared for probing as described by Langdale (1994), except for the protease treatment in which sections were incubated for 30 min at 37°C in 50  $\mu g \mbox{ mL}^{-1}$  proteinase K (Sigma). Both antisense and sense probes were synthesized using a SP6/T7 digoxigenin RNA labeling kit (Boehringer Mannheim), according to the manufacturer's instructions. Hybridization of probes was at 50°C overnight in 0.3 M NaCl, 10 mM Tris, pH 6.8, 10 mM NaPO<sub>4</sub>, 5 mM EDTA, 1  $\times$  Denhardt's solution (0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 1 mg mL<sup>-1</sup> tRNA (type XXI; Sigma), 10% dextran sulfate, and 40% formamide. After hybridization, sections were washed in 2  $\times$  SSC for 15 min at 20°C, RNase treated (20  $\mu$ g mL<sup>-1</sup> RNase, 0.5 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA) at 37°C for 30 min, and washed again in 2  $\times$  SSC (twice for 15 min) at 50°C, with a final wash in  $0.1 \times SSC$  for 15 min at 50°C. Detection of hybrids was performed using the digoxigenin nucleic acid detection kit (Boehringer Mannheim), following the manufacturer's instructions, except that 0.3% Triton X-100 was included in blocking, antibody, and wash solutions. Detection of hybrids (substrates were nitro blue tetrazolium salt, 5-bromo-4-chloro-3-indolyl phosphate, and toluidinium salt) was performed at 20°C for 16 hr in the dark. Sections were then dehydrated through an ethanol series (30, 50, 70, 90, and 100% ethanol, for 2 min in each) and washed

twice for 5 min in Histoclear (National Diagnostics, Atlanta, GA) before mounting in DPX mounting medium (Agar Scientific, Stanstead, UK).

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