

# PROLIFERATING INFLORESCENCE MERISTEM, a MADS-Box Gene That Regulates Floral Meristem Identity in Pea<sup>1</sup>

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*SQUAMOSA* and *APETALA1* are floral meristem identity genes from snapdragon (*Antirrhinum majus*) and Arabidopsis, respectively. Here, we characterize the floral meristem identity mutation *proliferating inflorescence meristem* (*pim*) from pea (*Pisum sativum*) and show that it corresponds to a defect in the *PEAM4* gene, a homolog of *SQUAMOSA* and *APETALA1*. The *PEAM4* coding region was deleted in the *pim-1* allele, and this deletion cosegregated with the *pim-1* mutant phenotype. The *pim-2* allele carried a nucleotide substitution at a predicted 5' splice site that resulted in mis-splicing of *pim-2* mRNA. PCR products corresponding to unspliced and exon-skipped mRNA species were observed. The *pim-1* and *pim-2* mutations delayed floral meristem specification and altered floral morphology significantly but had no observable effect on vegetative development. These floral-specific mutant phenotypes and the restriction of *PIM* gene expression to flowers contrast with other known floral meristem genes in pea that additionally affect vegetative development. The identification of *PIM* provides an opportunity to compare pathways to flowering in species with different inflorescence architectures.

The transition from the vegetative to the reproductive phase in plants commences when a signal from the leaves evokes a response in the shoot apical meristem that results in the development of flowers. The genes regulating the cascade of processes that occur in the shoot apex after this switch to reproductive growth have been well studied in the herbaceous species snapdragon (*Antirrhinum majus*) and Arabidopsis (Simpson et al., 1999; Theissen, 2001). For example, in snapdragon, the floral meristem identity gene *SQUAMOSA* (*SQUA*) is required for the transition to flowering and floral organ specification. This was determined by the phenotypes of *squa* null mutants, which typically produce reiterated inflorescences in place of flowers (Huijser et al., 1992). Flower formation, when it occurs, includes a wide range of floral abnormalities, especially in the two outer whorls (Huijser et al., 1992), suggesting that

*SQUA* also functions in first- and second-whorl organ specification in snapdragon.

In Arabidopsis, a corresponding role in floral development is carried out by *APETALA1* (*API*). Flowers on Arabidopsis plants carrying strong *ap1* mutant alleles retain many inflorescence-like characteristics; first-whorl organs are converted into bract or leaf-like organs bearing axillary flowers, which then repeat the pattern of the first flower (Irish and Sussex, 1990; Mandel et al., 1992; Bowman et al., 1993). The addition of a second mutation, *cauliflower* (*cal*), to an *ap1* mutant background completely transforms the aberrant flowers into proliferating inflorescences, although the *cal* mutation has no effect in a wild-type (*API*) background (Bowman et al., 1993). This double mutant phenotype implicates *CAL* in the acquisition of floral meristem identity and suggests that it has a redundant role with *API* in this process. The functional redundancy of *API* and *CAL* reflects their close molecular relationship; both are members of the same MADS-box gene subclade (Kempin et al., 1995; Theissen et al., 2000).

*API* is transcribed in response to light treatments (Hempel et al., 1997) and the flowering time gene, *CONSTANS* (Simon et al., 1996); thus, it acts as a molecular marker for floral determination (Hempel et al., 1997). It is also transcriptionally activated by another floral meristem identity gene, *LEAFY* (*LFY*; Parcy et al., 1998; Wagner et al., 1999). Although *LFY* acts non-cell autonomously in floral specification, *API* activates target genes in a mainly cell autonomous manner (Sessions et al., 2000). Transgenic ex-

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periments demonstrated that target genes of AP1, such as *APETALA3* (Hill et al., 1998), are activated via the formation of ternary and quaternary complexes of MADS-box proteins in Arabidopsis (Honma and Goto, 2001). Corroborating in vitro experiments with *SQUA* showed that it binds to promoter motifs in multimeric complexes, together with other MADS-box proteins, including DEF and GLO (Egea-Cortines et al., 1999). It was suggested that the combinations of proteins within these complexes provides regulatory specificity during floral development (Egea-Cortines et al., 1999; Honma and Goto, 2001). Thus, a detailed picture of a hierarchy of genes regulating floral meristem specification and development is emerging. Identification of homologous mutations in crop species will help to indicate the extent to which gene activities uncovered in these model species have diversified or been conserved.

The phenotype of the *proliferating inflorescence meristem* (*pim*) mutant from pea (*Pisum sativum*) is similar to that of *squa* and *ap1* mutants, and it was suggested that *PIM* may represent a floral meristem identity gene (Singer et al., 1994). A good candidate for the gene corresponding to *PIM* is *PEAM4*, a MADS-box gene that is closely related to *AP1*, *CAL*, and *SQUA*. *PEAM4* has been shown to rescue floral organ defects in the *ap1-1* mutant of Arabidopsis when expressed in transgenic plants under the control of the 35S promoter (Berbel et al., 2001). *PEAM4* expression was altered in the pea floral homeotic mutants *calix carpellaris* and *frondosus* (Berbel et al., 2001), but the allelic relationship between these mutations, *pim*, and *PEAM4* has not been investigated. In this paper, we describe two *pim* mutations that result in delayed floral meristem specification and first- and second-whorl floral abnormalities, and we show that both *pim* mutants carry altered *PEAM4* alleles, one of which results in aberrant transcript splicing.

## RESULTS

### Wild-Type Pea Inflorescence Structure

The inflorescence of pea has been described as a raceme (Hole and Hardwick, 1976) and a panicle (Tucker, 1989). Current interpretations agree that the main shoot apex is converted into a primary inflorescence on floral induction, and this primary inflorescence bears morphologically distinct secondary inflorescences that terminate in a hairy stub after producing one or two flowers (Singer et al., 1994; Ferrándiz et al., 1999). The primary inflorescence bears compound leaves and is indistinguishable from the vegetative shoot from which it is derived, apart from the production of secondary inflorescences (Makasheva, 1983). This inflorescence architecture is illustrated schematically in Figure 1A. Pea flowers are typical of the Papilionoideae, with five green sepals fused at the base, forming a cup, and five colored petals differentiated into three petal types.

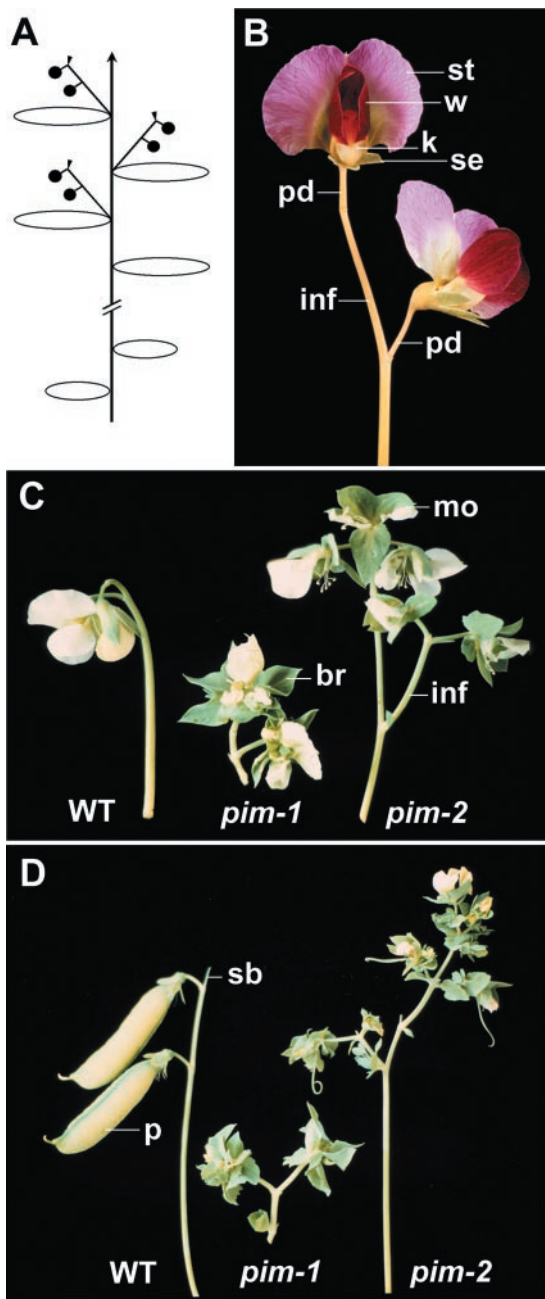
The standard is the largest and uppermost and there are two wings laterally and two fused petals that form the keel (Fig. 1B). Enclosed within the keel are 10 stamens, nine fused and one free, which surround the single, central carpel (Tucker, 1989; Ferrándiz et al., 1999).

### *pim* Mutations Delay Floral Meristem Specification and Alter Floral Morphology

A spontaneous, recessive mutant was identified in Minnesota and named *pim-1* after its severe floral abnormalities (Singer et al., 1994). A second, spontaneous mutant with a similar phenotype was identified in Tasmania. This latter mutation segregated in accordance with a 3:1 ratio ( $P > 0.5$ ) from a cross to its wild-type progenitor line, indicating that it was controlled by a single recessive allele. Allelism between *pim-1* and the Tasmanian mutant (*pim-2*) was confirmed by crosses between a plant heterozygous for *pim-2* and a homozygous *pim-1* plant (HL 244): Five of seven  $F_1$  plants produced mutant flowers. Comparison of *pim-2* plants with their isogenic wild-type siblings failed to reveal any significant differences in vegetative traits, such as length of basal internodes and the nodes where leaflet number increased; likewise, the node where the first secondary inflorescence occurred was not altered ( $P > 0.5$  for all traits). This analysis indicated that the *pim-2* mutation specifically affected flower development.

Primary and secondary inflorescences were correctly specified in both *pim-1* and *pim-2* mutants, but the transition from secondary inflorescence to flower production was delayed. In place of floral meristems, additional secondary-like inflorescences were produced (Fig. 1, C and D). Eventually, each of these inflorescences bore two or more abnormal flowers. *pim* mutants occasionally showed a form of floral reversion with a leafy shoot replacing one of the flowers on the secondary inflorescence. These leafy shoots seemed to represent a reversion to primary inflorescence, rather than vegetative development, because they bore aberrant flowers, as described below.

Floral morphology of *pim* mutants was aberrant in that first-whorl sepals were replaced by leafy bract-like structures, and second- and third-whorl organs were either absent or mosaic (Fig. 1, C and D). Early flowers on *pim-1* plants often consisted of these bracts surrounding the reproductive whorls; petals were entirely absent. Flowers consisting of outer bracts, petals, and a cluster of central stamens were also noted, as were complex, proliferating flowers, composed of combinations of the simpler flower types. Later flowers on *pim-1* and all flowers on *pim-2* produced morphologically normal standard and wing petals; however, petal position was irregular, and some flowers produced more than one standard or more than two wing petals. Wild-type flowers



**Figure 1.** Wild-type and mutant inflorescences. A, Schematic diagram of a wild-type pea plant. The center line with an arrow represents the abbreviated (//) main axis of the pea plant with its indeterminate apical meristem. At first, the apical meristem is vegetative and produces leaves (ellipses). On floral induction, the apical meristem is converted to an indeterminate primary inflorescence apex that bears secondary inflorescences (I) in the leaf axils. These in turn bear one or two flowers (●), and terminate in a stub (▼). B, Secondary inflorescence from a wild-type pea bearing two flowers. These are typical pea flowers with wild-type anthocyanin pigmentation, showing standard (st), two wings (w), and two fused petals forming the keel (k). Within the keel are the 10 stamens and a central carpel. Also visible are some of the five sepals (se), which form a cup surrounding the petals. The stub that terminates the secondary inflorescence (inf) is not visible behind the pedicel (pd). C, Young (at anthesis) secondary inflorescences from wild-type (WT), *pim-1*, and *pim-2* flowers from

**Table 1.** Floral organs present in flowers from the secondary inflorescence at reproductive node 8 of wild-type and *pim* mutant plants

	Genotype		
	Wild type	<i>pim-1</i> <sup>a</sup>	<i>pim-2</i> <sup>a</sup>
Flowers <sup>b</sup>	1	2.83 ± 0.28	2.83 ± 0.07
Leafy shoot	0	1.14 ± 0.14	0 ± 0.00
Bracts	0	5.86 ± 1.34	1.23 ± 0.20
Sepaloid bracts	0	4.00 ± 0.69	3.18 ± 0.33
Sepals	5	3.00 ± 0.96	4.40 ± 0.45
Petaloid sepals	0	4.29 ± 0.67	2.60 ± 0.28
Petals	5	4.71 ± 1.30	5.35 ± 0.44
Staminoid petals	0	4.86 ± 1.29	1.85 ± 0.16
Stamens	10	3.28 ± 0.81	12.83 ± 0.45
Carpeloid stamens	0	1.20 ± 0.17	0.20 ± 0.06
Carpels	1	0.14 ± 0.14	1.95 ± 0.10

<sup>a</sup> Seven *pim-1* and 20 *pim-2* flowers were examined. Values are mean no. of organs present at each position normally occupied by a single flower on a wild-type secondary inflorescence (±SE). <sup>b</sup> The no. of flowers on distinct pedicels present at each point normally occupied by a single flower in wild-type plants.

develop a single standard and two wings. Normal stamens and a single central carpel were seen in many flowers, although fusion of the carpel margins was not always complete. Self-pollination was uncommon in both *pim-1* and *pim-2* plants. Flowers produced late on the primary and lateral shoots often had a simpler structure, approaching wild type in appearance, except that the five sepal-like organs of the outer whorl were larger and leafier than those of wild-type flowers. These flowers also tended to produce fewer petals and stamens than wild type. To illustrate the extent of floral abnormality, counts were made of organs found on the secondary inflorescence of the eighth flowering node of *pim-1* and *pim-2* mutants, and these are listed in Table I.

#### The Relationship between *pim* and the *PEAM4* Gene from Pea

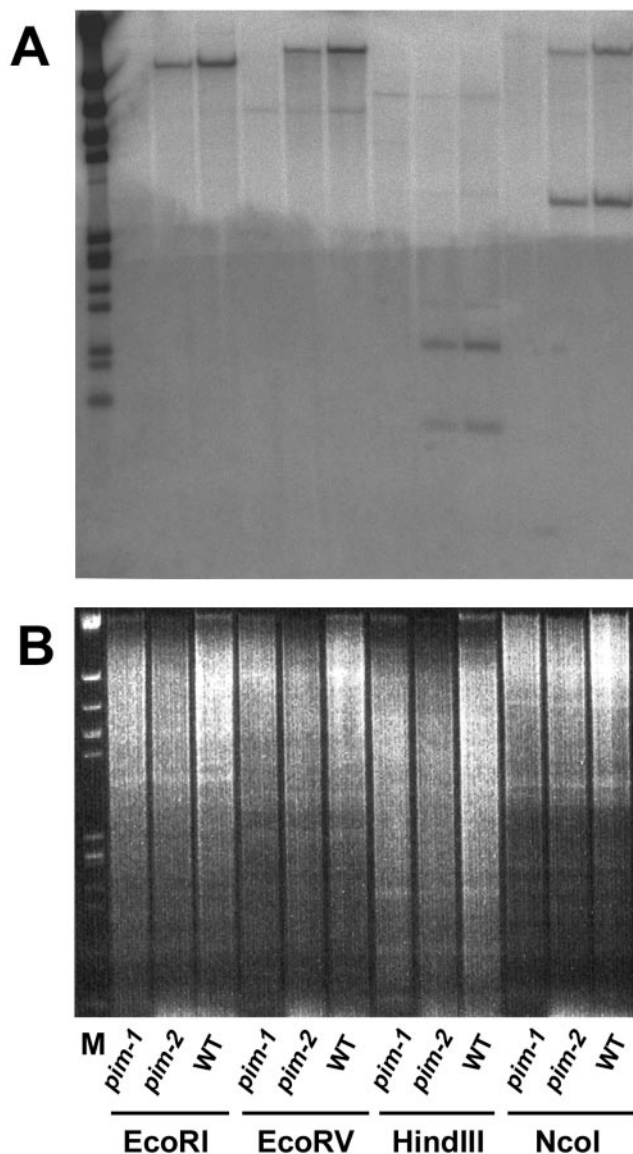
Because the phenotypes of the *pim-1* and *pim-2* mutants resembled those described for *squa* and *ap1*, a pea homolog of these genes was isolated from a shoot-tip cDNA library using the snapdragon *SQUA* gene (Huijser et al., 1992) as a probe. A full-length cDNA of 1,207 bp (GenBank accession no. AF461740), called *PEASQUA*, was mapped to linkage group IV of pea, using an *EcoRI* RFLP that segregated in a

plants with a white-flowered (anthocyanin absent) background. In the *pim* mutants, each flower is replaced by additional secondary inflorescences (inf) that bear abnormal flowers. Flowers are surrounded by leafy bracts (br) but are able to produce some petals, stamens, and carpels. Mosaic organs are also produced (mo). D, Secondary inflorescences from the same genotypes approximately 3 weeks later. A terminal stub is visible on the wild-type inflorescence (sb). The carpel of each wild-type flower has developed into a pod (p), and the proliferation of the *pim* mutant inflorescences has continued.

recombinant-inbred-line population derived from the cross JI 281  $\times$  JI 399 (Hall et al., 1997). *PEASQUA* was found to be 99% identical to *PEAM4*, an independently isolated pea homolog of *AP1* and *SQUA* (Berbel et al., 2001), although it is 10 bp longer than *PEAM4* in the 5'-untranslated region and 63 bp longer in the 3'-untranslated region. There are only two single-base mismatches within the coding regions when the sequences are aligned with each other, but these do not result in differences between the amino acid sequences. Given this degree of similarity, these *SQUA* homologs probably represent alleles of the same gene. The absence of a farnesylation motif at the 3' end of the open reading frame (Berbel et al., 2001) was confirmed in the *PEAM4* cDNA we isolated.

DNA gel blots of *pim-1*, *pim-2*, and wild-type plants, probed with the *PEAM4* cDNA minus the MADS-box region and washed at low stringency, were carried out to ascertain gene copy number and to compare the structures of the mutant and wild-type alleles, as shown in Figure 2A. There is one *NcoI* site in the *PEAM4* cDNA, and only two strongly hybridizing bands were observed in the *pim-2* and wild-type lanes on the DNA gel blot. This suggests that *PEAM4* is not duplicated in the genome, unless the duplicated copy has identical flanking and internal restriction enzyme sites. There are two *HindIII* sites in the *PEAM4* cDNA, and two strongly hybridizing bands were observed on the *pim-2* and wild-type lanes of the blot. Weaker hybridizing bands were also observed, one of which is predicted to produce a weak signal because it hybridizes to only 159 bp of the probe; the others probably represent a closely related gene. Apart from the single strongly hybridizing bands in the *EcoRI* and *EcoRV*-digested lanes, which again provide support for a single-copy gene, a faintly hybridizing band can also be seen in *pim-1* and *pim-2* mutant lanes and wild-type lanes, which is likely to represent a closely related gene.

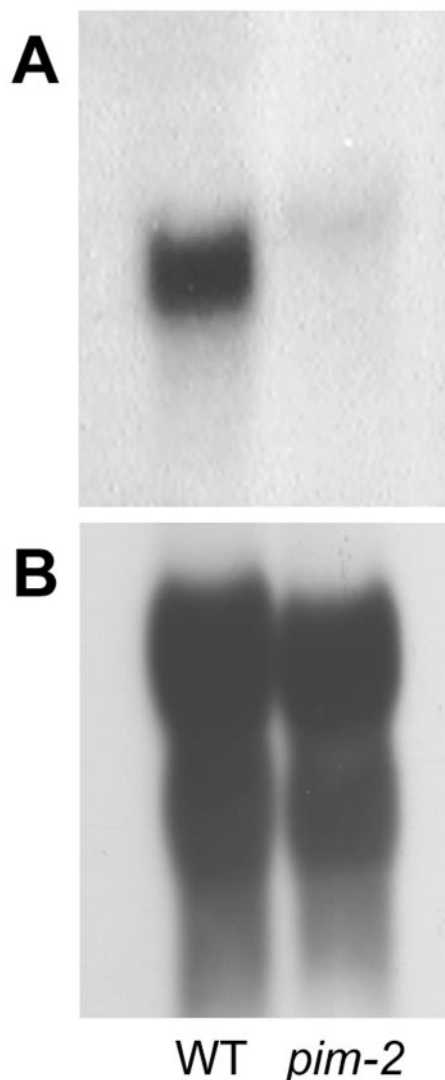
No hybridization signals were detected in the lanes corresponding to *pim-1*, although the ethidium bromide-stained gel (Fig. 2B) confirmed that all lanes were equally loaded with digested DNA. The absence of both bands in the *NcoI*-digested *pim-1* lane indicated that a deletion of the entire *PEAM4* coding region had occurred in the *pim-1* mutant line. This deletion cosegregated with the *pim-1* mutant phenotype (data not shown), consistent with *PEAM4* corresponding to *PIM*. It was possible, however, that the deletion in *pim-1* mutants was large, encompassing other genes besides *PEAM4*. To substantiate further the possible correspondence between *PEAM4* and *PIM*, the *pim-2* allele was examined. No differences could be detected between wild type and *pim-2* on DNA gel blots using restriction enzymes *BamHI* (data not shown), *EcoRI*, *EcoRV*, *HindIII*, and *NcoI* (Fig. 2A). On an RNA gel blot probed with *PEAM4*, shown in Figure 3A, the hybridizing transcript from *pim-2* mu-



**Figure 2.** DNA gel-blot analysis. A, *EcoRI*, *EcoRV*, *HindIII*, and *NcoI*-digested *pim-1*, *pim-2*, and wild-type (WT) genomic DNA, probed with the C-terminal fragment of the *PEAM4* cDNA and washed at low stringency. B, Ethidium bromide-stained gel of the samples shown in A, before they were blotted to a filter. Marker lane (M) contained bacteriophage lambda DNA digested with *EcoRI* and *HindIII* to generate 14 fragments, 21, 9.4, 6.6, 5.0, 4.3, 3.6, 2.3, 2.0, 1.9, 1.6, 1.4, 0.9, 0.8, and 0.6 kb in size.

tant flowers was larger and less abundant than that seen in similarly aged wild-type flowers. The difference in transcript abundance was assessed on the basis that the wild-type and *pim-2* mutant lanes were approximately equally loaded with RNA when the gel blot was reprobed with an rDNA probe (Fig. 3B). This indicated that the *pim-2* mutation disrupted *PEAM4* gene expression and further confirmed their identity.

To characterize the aberrant transcript in more detail, nested PCR was performed on reverse-transcribed cDNA templates, produced from wild-



**Figure 3.** RNA gel-blot analysis. A, Total RNA from *pim-2* and wild-type (WT) flowers, probed with the C-terminal fragment of the *PEAM4* cDNA. B, Total RNA from *pim-2* and wild-type (WT) flowers, probed with rDNA.

type and *pim-2* flowers, using oligonucleotides specific to the *PEAM4* sequence. Only one PCR product was amplified from wild-type cDNA, but two *pim-2* PCR products were amplified: One was approximately 100 bp larger than wild type, and the second was approximately 100 bp smaller (not shown). Sequence analysis revealed a 104-bp insert in the larger *pim-2* PCR product that was not present in wild-type cDNA, as illustrated in Figure 4. Sequences flanking the insert were identical to those of the wild-type PCR product and the original cDNA clone. To examine the nature of this insert further, the region was amplified from wild-type and mutant genomic DNA using PCR. The aligned sequences confirmed that the insert was present in both wild-type and mutant genomic DNA PCR products (data not shown). The insert was AT rich (76%; see Fig. 4), which is charac-

teristic of plant introns (Goodall and Filipowicz, 1989), and the position of this putative intron was consistent with the position of the fourth intron present in *SQUA* and *AP1* genomic sequences (Huijser et al., 1992; Mandel et al., 1992). It is significant that wild-type genomic DNA sequence differed from the *pim-2* sequence by a single-base change, substitution of an adenine for a guanine, at the predicted 5' splice site (Fig. 4); thus, the presence of the 104-bp insert in *pim-2* mRNA was probably a consequence of a failure in splicing. The resulting *pim-2* mutant open reading frame is predicted to terminate with a stop codon three bases after the A for G substitution. The low abundance of this larger transcript relative to wild type (Fig. 3A) suggests that the unspliced transcript may be less stable than the wild-type transcript.

Sequence from the smaller PCR product amplified from *pim-2* cDNA revealed a 100-bp deletion relative to wild type that removed the predicted exon between the predicted third and fourth intron positions. This mis-splicing by exon skipping (removing the third intron, intervening exon, and fourth intron) would result in a frame shift that would terminate translation at a stop codon 16 amino acids after the splice junction. Other intron sites, whose approximate positions were predicted from the conserved intron positions in *SQUA* and *AP1*, were correctly spliced in the *pim-2* mutant cDNA, and no additional sequence differences were detected in the PCR products obtained using cDNA from wild-type and mutant plants.

Together, the results of this molecular analysis of *PEAM4* alleles present in two independent *pim* mutants strongly supports the identity of *PEAM4* and *PIM*: the *pim-1* allele corresponding to a gene deletion and the *pim-2* allele corresponding to a single-base change that results in aberrant transcript splicing.

#### Expression Pattern of *PIM*

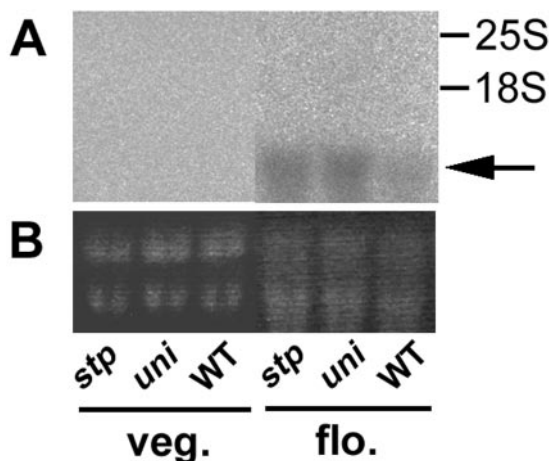
The phenotype of the two *pim* mutants suggested a role for *PIM* during floral meristem development, therefore, we examined the expression pattern of *PIM* in shoot tips before and after flowering as shown in Figure 5. *PIM* expression was not detected in vegetative shoot tips, but was detected in flowering shoots of all three genotypes examined (Fig. 5A). The mutants *unifoliata* (*uni*) and *stamina pistilloida* (*stp*), which correspond to *lfy* and *unusual floral organs* (*ufo*) in *Arabidopsis* (Hofer et al., 1997; Taylor et al., 2001), were included in this analysis to investigate whether *PIM* expression was dependent on *UNI* or *STP*. *PIM* expression in flowering shoots was not dependent on *UNI* or *STP* (Fig. 5A). In both mutants, the level of *PIM* expression was higher than in wild type, as assessed by the approximately equal amounts of RNA loaded in each gel lane (Fig. 5B).

	1			▼		50				
WT genomic	TCAA	ACTCAT	TCGT	ACACGC	AGAG	TATAAT	CTCT	TTTTCTT	CACT	TAACTA
<i>pim-2</i> cDNA	TCAA	ACTCAT	TCGT	ACACGC	AGA	<b>A</b> TATAAT	CTCT	TTTTCTT	CACT	TAACTA
WT cDNA	TCAA	ACTCAT	TCGT	ACACGC	AGA	.....	.....	.....	.....	.....
	51									100
WT genomic	TACT	GTGTTT	CTT	GGTTTCTC	ATCA	TACATTA	TTGA	ACTAGT	GTA	AATTTTC
<i>pim-2</i> cDNA	TACT	GTGTTT	CTT	GGTTTCTC	ATCA	TACATTA	TTGA	ACTAGT	GTA	AATTTTC
WT cDNA	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	101									150
WT genomic	ATT	TACTTG	ATAT	TTTAAAT	TAA	ACAGAAC	CAAC	TATGT	ACG	AGTCCAT
<i>pim-2</i> cDNA	ATT	TACTTG	ATAT	TTTAAAT	TAA	ACAGAAC	CAAC	TATGT	ACG	AGTCCAT
WT cDNA	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

**Figure 4.** Sequence analysis of PCR products. Alignment of sequences from *PEAM4* PCR products from wild-type (WT) genomic DNA and from *pim-2* and wild-type cDNA. A single guanine to adenine substitution at the 5'-splice acceptor site is highlighted in bold (arrowhead).

We examined *PIM* expression in floral tissues in more detail by RNA in situ hybridization analysis. *PIM* had a clearly delineated pattern of expression within developing floral primordia, as illustrated in Figure 6, and expression was not observed in vegetative tissue or mature inflorescences. *PIM* expression occurred throughout the entire floral primordium at stage 2 (Fig. 6A, flower F1), as defined by Ferrándiz et al. (1999). Later, during stage 4 of floral ontogeny, *PIM* expression was limited to the outer two whorls that were initiating sepal and common petal/stamen primordia, but expression also extended downward into the pedicel of the developing flower; the central carpel dome clearly lacked the hybridization signal (Fig. 6B, flower F1). The location of *PIM* expression within common primordia at stage 4 marked the identity of organs subsequently initi-

ated during stage 5, because expression was present in petal-fated cells but absent from stamen-fated cells (Fig. 6B, flower F1). At stage 5 and later, *PIM* expression was restricted to sepals (Fig. 6B, flower F2) and petals (Fig. 6C). This pattern of expression confirms the observations of Berbel and colleagues (2001) and is very similar to the expression patterns of *AP1* (Mandel et al., 1992) and *SQUA* (Huijser et al., 1992) during the development of Arabidopsis and snapdragon flowers. The absence of *PIM* expression in vegetative tissues (Fig. 5) was confirmed in the *afila* genotype, where the *UNI* gene is known to be highly expressed in developing leaves (Gourlay et al., 2000). In this longitudinal section, *PIM* expression was clearly confined to the floral primordia and absent from subtending leaves (Fig. 6D).



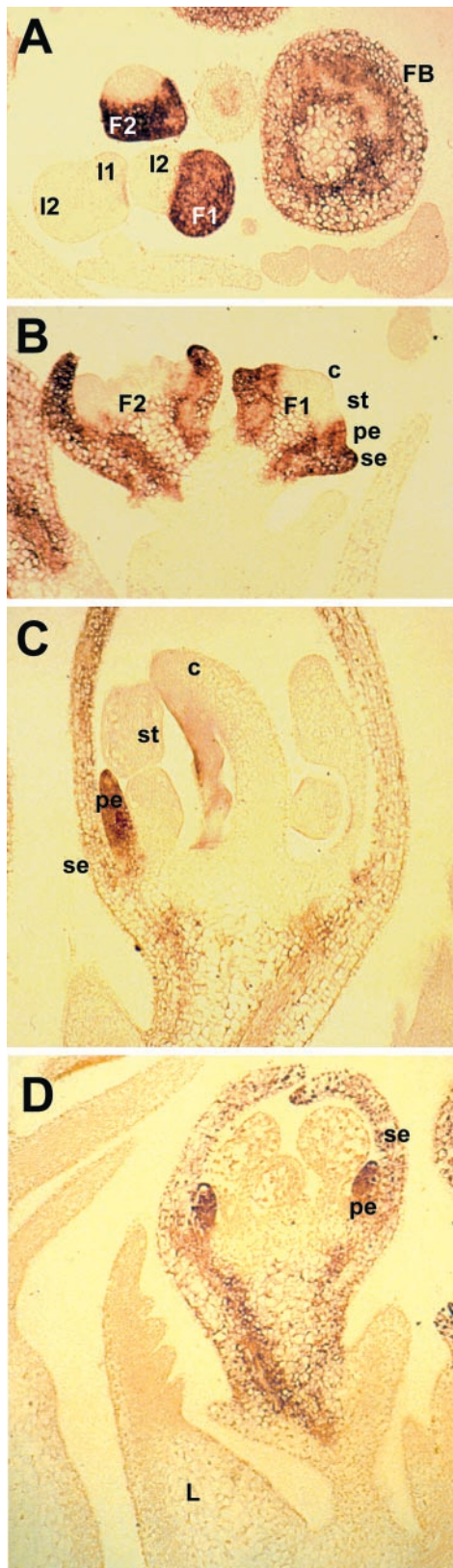
**Figure 5.** *PEAM4* expression in *uni* and *stp* mutants before and after flowering. A, Northern gel blot using total RNA from sibling *uni*, *stp*, and wild-type (WT) plants, probed with the C-terminal fragment of the *PEAM4* cDNA and washed at 65°C in 0.5× SSC. The first three lanes contain RNA from plants in the vegetative phase, the last three lanes contain RNA from flowering plants. The positions of the 25S and 18S ribosomal RNA bands are shown on the right. The *PEAM4* transcript is indicated (arrow). B, Ethidium bromide-stained gels of the samples shown in A, before they were blotted to a filter.

## DISCUSSION

### Comparative Flower Development

Mutations in *SQUA* homologs have been characterized so far in only two species, snapdragon and Arabidopsis. The identification here of *PIM* as a homolog of *SQUA* and *AP1* provides an opportunity to extend our understanding of the role of these genes in floral meristem specification to a third species, pea. It also enables a more complete comparison of the pathways to flowering proposed for pea (Weller et al., 1997) with those proposed for Arabidopsis (Piñeiro and Coupland, 1998; Simpson et al., 1999) and those being investigated in snapdragon (Cremer et al., 1998).

The similarity between the *pim* mutant phenotype and those of *squa* and *ap1* implies a conservation of gene activity. Mutations in *SQUA* and *AP1* result in a reiterating inflorescence phenotype (Irish and Sussex, 1990; Huijser et al., 1992; Mandel et al., 1992; Bowman et al., 1993) that is analogous to the replacement of flowers by proliferating secondary inflorescences and primary inflorescence-like leafy shoots seen in *pim* mutants. Conservation of *AP1* and *PIM* gene function is also supported by transgenic exper-



**Figure 6.** RNA in situ analysis of *PEAM4* expression in developing pea flowers. A, *PEAM4* expression in wild-type genotype HL 107 was confined to the flower and was not detected in vegetative or inflorescence tissue (I1 and I2 indicate the primary and secondary inflorescences, respectively). Expression occurred throughout young floral primordia (flower F1) at stage 2 and was also apparent in the oblique section through flower 2 (F2) and in a transverse section through the base of an older flower bud (FB) at approximately stage 7 of development. B, Stage 4 (F1) of development in genotype HL 107, showing *PEAM4* expression in the petal region of the petal/stamen common primordia (pe) and the sepals (se). Expression was absent from the stamen region of the petal/stamen common primordia (st) and the carpel primordium (c). Stage 5 (F2) flower showing expression in the outer sepal whorl. Petals express *PIM* at this stage but were not in the plane of this section. C, Stage 7 flower bud of genotype HL 107 showing *PEAM4* expression limited to sepals and petals and absent from stamens (st) and the carpel. D, Stage 7 flower bud of the *afila* genotype, JI 1195, showing *PIM* expression limited to the two outer whorls and absent from the subtending compound leaf (L). Magnification  $\times 75$  in A through D.

iments in Arabidopsis. *PIM* (*PEAM4*) overexpression in an *ap1* mutant partially complemented the mutation, and overexpression in a wild-type background mimicked *AP1* overexpression in that it resulted in early flowering (Berbel et al., 2001).

Although the proliferating inflorescence phenotype is in common, flower formation also occurs in *squa*, *ap1*, and *pim* mutants, suggesting that there is a redundant factor that can provide floral meristem identity in all three species. In Arabidopsis, redundant genes providing this function have been identified. For example, the role of *CAL*, which is very similar in sequence to *AP1*, was unmasked in an *ap1 cal* double mutant, because the *cal* mutation alone has no observable mutant phenotype (Bowman et al., 1993; Kempin et al., 1995). The flowers produced by *squa* and *pim* mutants are sometimes almost normal, in that they contain all organ types. In contrast, an entire complement of normal floral organs has not been observed on single-mutant *ap1* flowers; even on plants carrying weak alleles, sepals, and wild-type numbers of petals, are not seen (Irish and Sussex, 1990; Bowman et al., 1993). This suggests that, unlike snapdragon and pea, a redundant factor providing *AP1* function in outer whorl organ specification is absent in Arabidopsis.

Flowers with mosaic, or altered numbers of stamens and carpels, were observed on *pim* mutants (Table I), suggesting that *PIM* may have a role in the inner whorls of the flower. The nature of this role is difficult to clarify at present, because inner whorl organ numbers and types were not consistently altered in *pim-1* and *pim-2* mutants (Table I), which differ in their genetic backgrounds. Increased numbers of stamens and carpels and petaloid stamens were also described in *squa* mutants (Huijser et al., 1992), whereas reduced numbers of stamens, petaloid stamens, and incompletely fused carpels were reported in *ap1* mutants (Bowman et al., 1993). In snapdragon, Arabidopsis, and pea, the two outer whorls of mutant flowers are more strongly affected than the inner whorls (Huijser et al., 1992; Bowman et al., 1993; this work). Given that the ontogeny of a pea flower differs greatly from

rescences, respectively). Expression occurred throughout young floral primordia (flower F1) at stage 2 and was also apparent in the oblique section through flower 2 (F2) and in a transverse section through the base of an older flower bud (FB) at approximately stage 7 of development. B, Stage 4 (F1) of development in genotype HL 107, showing *PEAM4* expression in the petal region of the petal/stamen common primordia (pe) and the sepals (se). Expression was absent from the stamen region of the petal/stamen common primordia (st) and the carpel primordium (c). Stage 5 (F2) flower showing expression in the outer sepal whorl. Petals express *PIM* at this stage but were not in the plane of this section. C, Stage 7 flower bud of genotype HL 107 showing *PEAM4* expression limited to sepals and petals and absent from stamens (st) and the carpel. D, Stage 7 flower bud of the *afila* genotype, JI 1195, showing *PIM* expression limited to the two outer whorls and absent from the subtending compound leaf (L). Magnification  $\times 75$  in A through D.

snapdragon and Arabidopsis flowers, in that the second- and third-organ whorls are derived from a common primordium (Tucker, 1989), it is surprising that there are not more profound differences in the corresponding mutant phenotypes.

### PIM Is the Ortholog of SQUA

Identification of orthologous gene pairs is useful, not only for comparison of gene functions, but also because they provide definitive single-point comparisons in genetic map alignments between species pairs. Resolution of orthologous relationships among *SQUA*, *PIM*, *AP1*, and *CAL* based on sequence similarity is difficult because the presence of two or more *SQUA*-like genes in some species suggests that complex relationships exist between the subfamily members, with the possibility of multiple independent duplication events. For example, phylogeny reconstructions suggest that *CAL* may have originated after a gene duplication (Theissen et al., 2000). However, because *CAL* orthologs have not yet been identified in species outside the Brassicaceae, the relationship of *CAL* to other *SQUA* homologs remains unclear. For this reason, we use the more general term homolog when referring to members of the clade containing *SQUA*, *AP1*, and *CAL*. Despite this difficulty in determining orthology with Arabidopsis genes, we consider that *PIM*, the only representative from pea in this clade (<http://www.mpiz-koeln.mpg.de/mads/madstrees.html>), and *SQUA*, the only representative from snapdragon, are orthologous genes. This conclusion is supported by the fact that only one band was detected on DNA gel blots probed with *PEAM4*, therefore it is unlikely that a duplicated gene exists in pea.

Orthology relationships between genes may be reflected by their map positions, so we have compared the map positions of *AP1*, *CAL*, and *PIM* to clarify their relationship to each other. *AP1* and *CAL* are 53 cM apart on the same Arabidopsis chromosome, and *CAL* maps very close to *UFO* (<http://Arabidopsis.org/servlets/mapper>). The pea ortholog of *UFO*, *STP*, maps to linkage group VII (Taylor et al., 2001), whereas *PIM* maps to linkage group IV (as marker *PEASQUA*; Hall et al., 1997). *PIM* is, thus, more like *AP1* than *CAL* in that it is not closely linked to the pea *UFO* ortholog. This is consistent with the mutant phenotypes, which also suggest that *PIM* shares more in common with *AP1* than it does with *CAL*.

### The *pim-2* Mutation Affects Transcript Splicing

The deletion of *PIM* in *pim-1* plants suggests that *pim-1* is a null allele. It is likely that *pim-2* also represents a null allele, first because the incorrectly spliced *pim-2* transcripts are very low in abundance and are predicted to terminate the open reading frame prematurely, and second, because the *pim-1*

and *pim-2* mutants exhibit similar morphological defects. The similarity of the *pim-1* and *pim-2* mutant phenotypes, furthermore, suggests that the deletion in *pim-1* is not so large as to include closely linked genes with major developmental effects. It is important to note that differences between the *pim-1* and *pim-2* mutant phenotypes may not be allelic differences but may result instead from the different genetic backgrounds of these two mutants.

Northern gel-blot and sequence analysis of the *pim-2* allele indicate that the G to A transition results in the production of aberrant transcripts by failure to excise the fourth intron and by exon skipping. There are other cases of G to A mutations in the 5' splice sites of Arabidopsis introns where the effects of the mutations on splicing have been studied. For example, the transition present in the *Rubisco activase* mutant resulted in an accumulation of differently sized splicing intermediates that were detectable by northern gel-blot analysis (Orozco et al., 1993). The higher  $M_r$  *pim-2* transcripts we detected on northern gel blots were of a uniformly larger size than wild type and were thus likely to represent the intron 4-containing transcript that was also identified among the cloned *pim-2* cDNA products. A similar effect was observed in the *phytochrome B-103* mutant, where the major effect of the mutation was a failure to splice the intron (Bradley et al., 1995).

Another splicing behavior of the *pim-2* mutant, detected only among sequenced cDNA products, was exon-skipping. Exon 4, which lies 5' adjacent to the mutation, and both flanking introns, were excised. This was not reported for the *Rubisco activase* and *phytochrome B-103* mutations, but was the major defect caused by the G to A mutation in the 5' splice site of the *constitutive photomorphogenic1-2* allele (Simpson et al., 1998). The *pim-2* mutation, thus, provides further support for a role for exons, as well as introns, in pre-mRNA splice site definition (Simpson et al., 1998).

Both types of *pim-2* defective transcripts would lead to premature truncation of the C-terminal domain of the *PIM* open reading frame, which is required by *SQUA*, *DEF*, and *GLO* proteins for the formation of ternary complexes in yeast (*Saccharomyces cerevisiae*; Egea-Cortines et al., 1999). The low abundance of the transcripts relative to wild type suggests that they may be subject to mRNA surveillance-mediated degradation (Hilleren and Parker, 1999). If this type of degradation occurs, it is not possible to distinguish whether the complete absence of the exon-skipping transcript on northern gel blots is because it is subject to more rapid decay than the intron-retaining transcript, or because the exon-skipped transcript is a rarer aberrant splicing product in the mRNA pool.

### The Role of *PIM* in Pea Flower Development

*PIM* gene expression in developing flowers has been described recently and was found to be gener-



ally similar to the expression patterns of *AP1* and *SQUA* (Berbel et al., 2001). The early transcription of these genes within developing floral primordia (Huijser et al., 1992; Mandel et al., 1992) reflects their common roles in floral meristem specification. Later in floral development, differences are apparent. *SQUA* is expressed in the developing carpel, but expression is excluded from stamen primordia (Huijser et al., 1992), whereas in this work, we confirm that *PIM* expression is excluded from both inner whorls (Berbel et al., 2001), as is *AP1* (Mandel et al., 1992). Another difference is that *SQUA* is expressed in the bracts subtending flowers in snapdragon (Huijser et al., 1992), but not in Arabidopsis, where bracts are absent, nor in pea, where production of bracts in these genotypes is rare and unpredictable. However, these variations in patterns of gene expression do not seem to correlate with the minor differences in mutant phenotypes of the three species, such as the stronger effect of the *ap1* mutation on outer whorl organ identity, compared with *pim* and *squa*. Differences in expression patterns or mutant phenotypes may reflect differences in wild-type development between these three species. Different requirements for farnesylation may also contribute to species differences. *PIM* and genes homologous to *AP1* cloned from grass species (Gocal et al., 2001) do not contain a 3'-farnesylation sequence motif that is present in *AP1* and other members of the clade (Berbel et al., 2001).

Peas have more complex leaves and inflorescence architecture than do Arabidopsis and snapdragon, and for this reason pea is an interesting species in which to examine the functions of homologous genes. Two other floral meristem identity genes have been identified previously. These are *UNI*, the ortholog of *LFY* (Hofer et al., 1997), and *STP*, the ortholog of *UFO* (Taylor et al., 2001). Both of these have been shown to have wider roles in vegetative development, apart from their participation in floral meristem specification. In contrast, the role of *PIM* is specific to the flower, because other aspects of plant development are unaffected in *pim* mutant plants.

Steroid-inducible activation of *LFY* in transgenic Arabidopsis showed that *AP1* is directly transcriptionally regulated by *LFY* in inflorescences (Wagner et al., 1999). Although *LFY* was misexpressed throughout Arabidopsis plants using this inducible 35S promoter construct, *AP1* transcription was activated only in the tissues and at the stage when floral fate would normally be assumed in wild type (Wagner et al., 1999): *AP1* was not transcriptionally activated throughout the plant. Contrasting results were obtained by Parcy et al. (1998), who showed that activation of an *AP1::GUS* reporter gene occurred throughout transgenic 35S::*LFY* Arabidopsis seedlings before flowering. Our data suggest that tissue specificity in the activation of *AP1* by *LFY* is conserved in peas. In pea leaves, *UNI* expression alone

seems to be insufficient to up-regulate *PIM*, because *afila* mutant leaves, with prolonged and high levels of *UNI* expression (Gourlay et al., 2000), do not express *PIM* (see Fig. 6D).

Reports on the transcriptional activation of *AP1* by *LFY* also vary on whether *AP1* expression is reduced (Wagner et al., 1999), or almost normal (Parcy et al., 1998), in *lfy* mutants. In snapdragon, *SQUA* expression in the *floricaula* mutant is comparable with that of wild type (Huijser et al., 1992). Our results show that in pea, *PIM* expression is not reduced, but is increased, in a *uni* mutant background. The same result was obtained in a *stp* mutant background. Both of these mutations result in the production of flowers with supernumerary whorls of sepals and sepalloid organs (Hofer et al., 1997; Taylor et al., 2001). Thus, increased *PIM* expression relative to wild type is consistent with an increased number of first-whorl organs in the mutants. Our results clearly demonstrate that *PIM* expression is independent of *UNI* and *STP* during flowering.

Previous studies of *uni* have emphasized its unique leaf phenotype and its interactions with the leaf homeotic mutants in pea (Hofer et al., 1997; Gourlay et al., 2000; Taylor et al., 2001), rather than its role in floral specification. A detailed analysis of double mutants and their effects on flowering is now possible. These experiments and the identification of B- and C-class floral homeotic genes corresponding to *APETALA3*, *PISTILLATA*, and *AGAMOUS* are required to elucidate further the gene interactions in pea flower development and to determine the extent of conservation of gene function between Arabidopsis and pea.

## MATERIALS AND METHODS

### Plant Material and Cultivation

The pea (*Pisum sativum*) *pim-1* and *pim-2* mutations occurred spontaneously as independent events at Carleton College (Northfield, MN) and the University of Tasmania (Hobart, Australia), respectively. The *pim-1* mutant does not have an isogenic wild-type line. The *pim-2* mutation arose in cv Torsdag (line HL107), and phenotypic analyses of *pim-2* were carried out on plants segregating in a second backcross to this line. Line HL107 was also used as the source of wild-type DNA and RNA. Seed of the *pim-2* mutant line resulting from the second backcross was deposited into the Hobart germplasm collection as HL285. Sibling plants carrying *uni-2171* (Hofer et al., 1997) or *stp-4* (Taylor et al., 2001) mutant alleles or the corresponding wild-type alleles were used in northern gel-blot analyses. All siblings were short-statured *afila tendril-less* genotypes (Taylor et al., 2001). Shoot tips from plants at the vegetative phase of development were harvested 21 d after sowing, and flowers and shoot tips from flowering plants were harvested 33 d after sowing.

Plants used in the phenotypic analysis of *pim-1* and *pim-2*, allelism tests, and gel blots were grown in Hobart in a 1:1 (v/v) mix of vermiculite and dolerite chips topped with 2 to 3 cm of pasteurized peat-sand potting mix under an 18-h photoperiod. Plants used for additional phenotypic analysis, gel blots, and RNA in situ hybridization studies were grown at the John Innes Centre in John Innes number 1 potting mix with 30% grit, under a 16-h photoperiod. All plants received liquid fertilizer weekly.

## Molecular Analysis of *PIM*

The *PEAM4* cDNA, cloned into the *EcoRI* and *XhoI* sites of pBluescript (Stratagene, La Jolla, CA), was initially identified as *PEASQUA*, and was isolated by screening a pea flowering-shoot-tip cDNA library (Hofer et al., 1997) with a full-length *SQUA* clone provided by Peter Huijser (Max Planck Institute, Köln, Germany). For analysis of transcript splicing, cDNA was produced by reverse transcription from total RNA isolated from *pim-2* and wild-type (HL107) flowers just before anthesis. Two pairs of primers specific to the *PEAM4* sequence were used for nested PCR: first round, (5') GGG ACG AGC TCA AAC TCA CAC (3') and (5') GGA GTT CCT TCT AGT GAT AG (3'); second round, (5') AGG AGA GCT GGA CTT CTC AAG (3') and (5') CTA CCA AAC ATA TAT ATA AGC (3'), using cDNA as a template. Primers flanking the insert present in the *pim-2* cDNA (5', ATG GGA GAA GAT TTG GGT ACA ATG and 5', TTC TGA AGC TCT GAA ATG GAC TCG) were used to amplify fragments from *pim-2* and wild-type genomic DNA. Amplified fragments were either subcloned into pGEM-T easy vectors (Promega, Madison, WI) for sequencing, or purified using a Concert PCR purification system (Invitrogen, Carlsbad, CA) and sequenced directly. Sequencing was carried out using ABI big dye terminator technology (Applied Biosystems, Foster City, CA).

RNA *in situ* hybridization was performed as described previously (Hofer et al., 1997) on 8- $\mu$ m sections of wild-type flowering pea apices using digoxigenin-labeled sense and antisense probes. DNA and RNA blots and *in situ* hybridization analyses were performed using a modified clone that had the MADS-box region between restriction sites *EcoRI* and *SpeI* removed, to prevent cross hybridization with other MADS-box genes. Unless otherwise specified, high-stringency washes were at 65°C in 0.1 $\times$  SSC and low-stringency washes were at 50°C in 2 $\times$  SSC.

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