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Additional Information

PsPMEP, a pollen specific pectin methylesterase of pea (*Pisum sativum* L.)

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Running title: PsPMEP a pollen specific pectin methylesterase

Abstract

Pectin methylesterases (PMEs) are a family of enzymes involved in plant reproductive processes such as pollen development and pollen tube growth. We have isolated and characterized *PsPMEP*, a pea (*Pisum sativum* L.) pollen specific gene that encodes a protein with homology to PMEs. Sequence analysis showed that *PsPMEP* belongs to group 2 PMEs, which are characterised by the presence of a processable amino-terminal PME1 (inhibitory) domain followed by the catalytic PME domain. Moreover, several motifs highly conserved among PMEs contain the essential amino acid residues involved in enzyme substrate binding and catalysis. Northern blot and *in situ* hybridization analyses showed that *PsPMEP* is expressed in pollen grains from 4 days before anthesis till anther dehiscence and in pollinated carpels. In the *PsPMEP* promoter region we have identified three CARG boxes showing the consensus motif C[A/T]₈G, which is preferentially bound by the MADS domain of the transcriptional regulator AGAMOUS-like 15 (AGL15), as well as other conserved *cis* regulatory elements that have been associated to gene pollen specific expression. Expression analysis of *PsPMEP* promoter fused to the *uidA* reporter gene in *Arabidopsis thaliana* plants showed a similar expression pattern when compared with pea, indicating that this promoter is also functional in a non-leguminous plant. GUS expression was detected in mature pollen grains, during pollen germination and during pollen tube penetration and elongation along the transmitting tract and when the pollen tube reaches the embryo sac in the ovule.

PsPMEP GenBank accession n.: KC964536

Introduction

Pectin methylesterases (PMEs) produced by plants are involved in diverse physiological processes associated to both vegetative and reproductive development (Pelloux et al. 2007). Their roles in reproductive development have mainly concerned with pollen development and pollen tube growth (Jiang *et al.* 2005; Francis *et al.*, 2006; Tiang *et al.*, 2006; Bosch and Hepler; 2006). PMEs belong to family 8 (CE8) of carbohydrate esterases (<http://www.cazy.org/fam/CE8.html>). These enzymes catalyze the specific demethylesterification of the homogalacturonan (HGA) fraction, a major component of pectins, within plant cell walls, releasing methanol and protons, and creating negatively charged carboxyl groups in the process. The demethylesterified HGA can either form Ca²⁺ bonds, which promote the formation of the so-called 'egg-box' model structure, thus forming gels, or become a target for pectin-degrading enzymes, such as polygalacturonases, affecting the texture and rigidity of the cell wall. The enzymatic activity of PMEs can lead either to cell wall loosening or to cell wall stiffening, depending on the apoplastic pH and the availability of divalent cations, thereby affecting shape and growth of plant cells. Thus, PMEs have major roles in pectin remodelling *in muro*. PMEs belong to large multigene families and their primary and quaternary structures are conserved among plant taxa (Markowic and Janecek 2004).

Higher plant PMEs are frequently so-called pre-pro-proteins, in which the mature, active part of the protein (PME domain) is preceded by an N-terminal extension (pro-region) that varies in length and shows a relatively low level of amino acids identity between isoforms. The pre-region is required for protein targeting to the endoplasmic reticulum, while only the mature part of the PME, without the pro-region, is extracted from the cell wall. Although several functions for the pro-region have been suggested (Micheli 2001; Markowic and Janecek 2004; Pelloux et al. 2007; Jolie et al. 2010), including targeting of PME to the cell wall, correct folding of PME and inhibition of PME enzyme activity, none has been conclusively established. Transient expression studies with green fluorescent protein (GFP) fusion proteins suggest that the pro-region is important for the correct targeting of the mature PME to the apoplast and data from *in vitro* growth analysis further support the idea that this region also acts as an intramolecular inhibitor of PME activity (Bosch et al. 2005). Spatial and temporal regulation of PME activity during plant development is based on a large family of isoforms. The pro-region shares similarities with the PME inhibitor domain (PMEI domain; Camardella et al. 2000; Scognamiglio et al. 2003). PME inhibitors are thought to be key regulators of cell wall stability at the tip of the pollen tube. A classification has been created, based on the presence or absence of the PMEI domain: Arabidopsis PMEs of group 1 (250 to 400 amino acids; 27-45 kDa) have no PMEI domain; those of group 2 (500-900 amino acids; 52- 105 kDa) have 1-3 PMEI domains. These groups were formerly known as types II and I, respectively. Type-I PME genes contain one to three introns and the deduced proteins include a long pro-region, whereas type-II genes contain five or six introns and the pro-region of the deduced proteins is missing in most cases. The mature PMEs have similar structures and belong to the family of parallel β -helix proteins with major differences in loops making up the

1 substrate binding cleft. The active site of PME is located in the long shallow cleft lined by two
2 absolutely conserved aspartic acid residues in the center, Asp 136 and Asp 157 in the carrot
3 PME, the first 3D crystallographic structure obtained of a plant PME (Johanson et al. 2002;
4 Gummadi et al. 2007).
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7 Many of the characterized genes which are specifically expressed during pollen
8 development, pollen germination and pollen tube growth encode proteins that are likely to play a
9 role in cell wall metabolism, like is the case of PMEs. Earlier studies revealed that expression of
10 PME genes is strongly regulated in a tissue-specific manner (Li et al. 2002). The temporal
11 expression of such genes has been studied and they are found to be expressed late in
12 microsporogenesis reaching a maximum in mature microsporocytes. In some cases continued
13 expression in the pollen tube has also been demonstrated (Kononowicz et al. 1992; Bosch and
14 Hepler 2005). The genetic and molecular mechanisms that control the penetration of pollen tubes
15 through stigmatic and stylar tissues still poorly understood. The wall in the tip region of the pollen
16 tube is composed of a single pectin layer. The PMEs catalyze the demethylesterification of
17 homogalacturonans releasing acidic pectins and methanol, contributing to cell development by
18 regulating the mechanical and chemical properties of plant cell walls (Micheli 2001).
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25 Several studies have led to the identification of pollen tube-specific PMEs that could be
26 involved in the processes of pollen tube development and its interaction with female floral tissues
27 (Wakeley et al. 1998; Futamura et al. 2000; Li et al. 2002). Recent advances in our understanding
28 of PME functions in reproductive development have mainly concerned their roles in pollen
29 development and pollen tube growth demonstrated using reverse genetics. The first functional
30 analysis of a pollen tube-expressed PME was described by Jiang et al. (2005). *VANGUARD1*
31 (*VGD1*) is an *Arabidopsis thaliana* gene that encodes a PME-homologous protein required for
32 enhancing the pollen tube growth in the style and transmitting tract tissues. The *vgd1* mutation
33 resulted in defects of male gametophytic function (pollen tubes structurally unstable) but did not
34 affect female gametophytic function. Moreover, it has been shown in *Arabidopsis* that
35 *QUARTET1* (*QRT1*), which is expressed in pollen and surrounding anther tissues, has a role in
36 pollen tetrad separation during floral development and has PME activity when expressed in *E. coli*
37 (Francis et al. 2006). Rodríguez-Llorente et al. (2004) reported the presence of a gene family of
38 at least eight differentially expressed PMEs in the model legume *Medicago truncatula*. One sub-
39 family is represented by a single symbiotic gene (*MtPER*) that could participate in the infection
40 process during nodulation and two pollen-expressed genes that could be involved in pollen grain
41 development (*MtPEF1* and *MtPEF2*).
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51 Pollen specific PMEs are of special interest because their putative roles in pollen
52 maturation, germination and pollen tube growth. Here we show the isolation and characterization
53 of a pollen specific gene of pea (*PsPMEP*) which product shows homology to PMEs. The
54 *PsPMEP* promoter has different conserved *cis* regulatory elements putatively responsible of gene
55 pollen specific expression and three CArG boxes showing the consensus motif that is
56 preferentially bound by the MADS domain of the transcriptional regulator AGL15 (Tang and Pery
57 2003). The *PsPMEP* promoter fused to the *uidA* reporter gene shows specific GUS activity in the
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1 mature pollen grains, during pollen germination and in the growing pollen tubes of transgenic
2 *Arabidopsis thaliana* plants. Therefore, the *PsPMEP* promoter interestingly maintains its
3 expression pattern in a non-leguminous species and suggest that *PsPMEP* plays a role during
4 pollen development and pollen tube growth.
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7 8 **Materials and methods** 9

10 *Plant material*

11 *Pisum sativum* cv. Alaska plants were used in this study. Plants were grown in the greenhouse, at
12 22°C (day) and 18°C (night) with a 16 h light / 8 h dark photoperiod, in a mixture of soil:sand (3:1)
13 irrigated with Hoagland N^o.1 solution supplemented with oligoelements (Hewitt 1966). *Arabidopsis*
14 *thaliana* cv. Columbia (Nottingham *Arabidopsis* stock centre, UK) plants were grown in cabinets
15 at 21° C under long-day (16 h light) conditions, illuminated by cool-white fluorescent lamps (150
16 $\mu\text{mol quanta m}^{-2} \text{ sec}^{-1}$), in a (1:1:1) mixture of sphagnum : perlite : vermiculite and irrigated with
17 water and, once a week, with the same mineral solution.
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25 *Isolation and sequence analysis of the PsPMEP gene*

26 Poly(A)⁺ RNA obtained from pea pollen and pollinated stigmas was amplified by RT-PCR to
27 isolate cDNA fragments with homology to PME genes. For preparation of poly(A)⁺ RNA from total
28 RNA we used the Dynabeads mRNA purification kit (Invitrogen, <http://www.invitrogen.com>). RT-
29 PCR experiments were carry out using degenerated primers corresponding to two conserved
30 domains in almost all plant PMEs: GVYNE (5'-GGNGTNTAYAAYGAR-3') and YLGRPW (5'-CCA
31 NGGNCTTCCTARRTA-3'). We isolated a cDNA fragment corresponding to the expected length,
32 552 bp, which was cloned in the pGem-T-easy vector (Promega, <http://www.promega.es/>) and
33 sequenced. Later on, a screening of a pea genomic library generated in the vector EMBL4 using
34 partially *EcoRI* digested genomic DNA was performed, using the 552 pb fragment obtained by
35 RT-PCR as a probe. The hybridization and washings were carried out using standard procedures
36 (Sambrook et al. 1989). λ DNA from the positive plaques was extracted using a λ DNA extraction
37 kit (Qiagen, <http://www.qiagen.com>) and a 4.8 Kb λ DNA fragment containing the complete
38 *PsPMEP* gene was sequenced (GenBank accession n.: KC964536). Primers used for
39 sequencing are listed in supplementary table S1. The full-length ORF cDNA was isolated by RT-
40 PCR from Poly(A)⁺ RNA obtained from pea pollinated stigmas, using primers containing the start
41 and the stop codon (5'ATGGCAGAGGGTGGTGATGCAC-3' and 5'-
42 TCACCATGTAAGCACTGTCTTTTTG-3'), and cloned into the pGEM-T-easy vector (Promega,
43 <http://www.promega.es/>).
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55 Sequence alignment and similarity comparisons of the inferred protein were performed using the
56 MACVECTOR 9.5 software (MacVector, Inc., <http://www.macvector.com/>). The deduced amino
57 acid sequence was aligned using the CLUSTALW tool in MACVECTOR 9.5 and further refined by
58 hand. Secondary structure prediction of the mature protein was performed using the SOPMA
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1 application (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html;
2 Combet et al., 2000). Frequency distribution of different cis-regulatory elements in the *PsPMEP*
3 promoter was analyzed in the PLACE database (<http://www.dna.affrc.go.jp/PLACE/>).
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6 *Phylogenetic tree*

7 The phylogenetic tree was inferred by Neighbor-Joining using Poisson-corrected amino acid
8 distances. Reliability of internal nodes was assessed using bootstrap with 1000 pseudo-
9 replicates. Tree inference was conducted using MEGA version 4 (Tamura et al. 2007). The tree is
10 based on the alignment of conserved PME domains (Pfam01095) predicted with the SMART
11 program (<http://smart.embl-heidelberg.de/>).
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16 *Structure modelling*

17 The PSIPRED server (<http://bioinf.cs.ucl.ac.uk/psipred/>) was used to search for protein structures
18 suitable to be used as templates for modelling. Carrot pectin methylesterase (Johansson et al.
19 2002) (PDB id. 1GQ8) was chosen as the template for mature PsPMEP. The Swiss Model server
20 (<http://swissmodel.expasy.org/>) was used to generate the enzyme model. DeepView-Swiss-
21 PdbViewer (<http://spdbv.vital-it.ch/>) was used to analyse the structures and generate the figures.
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28 *Expression analysis of PsPMEP*

29 For Northern blot, root nodules, leaves, roots, stems and flower buds were collected, frozen in
30 liquid nitrogen and stored at -80°C. Total RNA (15 µg) was isolated using 500 µl of extraction
31 buffer (4% p-aminosalicylic acid, 1% 1,5 naphthalenodisulfonic acid) and 1 ml of phenol:chloroform
32 (1:1). The RNA was precipitated in 1/10 V of NaOAc 3M, 2V of EtOH 100% on ice. The resultant
33 pellet was dissolved in water and finally the RNA precipitation took place overnight at 4°C on an
34 equal volume of 8M lithium chloride. Total RNA was separated by electrophoresis in
35 formaldehyde-agarose gel, transferred to Hybond N⁺ membranes (Amersham Biosciences,
36 <http://www.gehealthcare.com/>), and hybridized with the ³²P-labelled 552 bp fragment under
37 standard conditions (Sambrook et al. 1989).
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39 RNA *in situ* hybridization with digoxigenin-labelled probes was performed on 8 µM longitudinal
40 paraffin sections of *Pisum sativum* flowers as described in Gómez et al. (2004) and on pollen
41 grains (whole-mount) as described in de Almeida Engler et al. (1998). Digoxigenin-labelled
42 antisense and sense RNA probes were synthesized from the 552 bp cDNA fragment of *PsPMEP*
43 cloned into the pGEM-T-easy vector (Promega, <http://www.promega.es/>), using the
44 corresponding T3 and T7 RNA polymerases and following the manufacturer's instructions
45 (Roche, <http://www.roche-applied-science.com/>). The RNA probes were hydrolyzed to 150 nt
46 before use in the hybridizations reactions. Control experiments were performed with the sense
47 probe and no significant signal was detected. Photographs were taken with a light microscope
48 (Eclipse E600 Nikon, <http://www.nikon.com>).
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59 *Chimaeric construct PsPMEP::uidA and production of transgenic A. thaliana plants*

1 Analysis of the *PsPMEP* promoter was carried out using a chimaeric construct in the vector
2 pBI101 containing the nopaline synthase constitutive promoter (nos-pro) fused to the *nptII* gene
3 (kanamycin resistance) and a 2138 bp HindIII/Sall fragment containing the 5' region upstream of
4 the *PsPMEP* start codon fused to the *uidA* gene (β -glucuronidase) and the polyadenylation signal
5 of the nopaline synthase gene (nos-ter) as terminator in the 3' ends of both genes (Fig. 5a).
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7 *Arabidopsis thaliana* cv. Columbia genetic transformation was performed by vacuum infiltration
8 and selection of the plants resistant to kanamycin as described Bechtold et al. (1993), using the
9 strain C58 of *A. tumefaciens*. The kanamycin resistant plants were transferred to pots with
10 vermiculite: perlite: peat (1: 1: 1) and grown in culture chambers at 22°C in long-day conditions
11 until the seeds were collected. Control plants were also transformed with the empty plasmid
12 pBI101.
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19 *β -glucuronidase (GUS) histochemical assay*

20 Transformed plants were submitted to histochemical analysis of the β -glucuronidase activity.
21 Vegetative and floral tissues were infiltrated using two vacuum pulses of 5 min in a GUS assay
22 buffer [0.1 M NaH₂PO₄, 10mM Na₂EDTA.H₂O, 0.5 M K₃Fe(CN)₆, 0.1% Triton X-100 and 0.3% 5-
23 bromo-4-chloro-3-indolyl β -D-glucuronide (X-Gluc)] and incubated in this solution at 37°C for 16
24 hours. Afterwards, de-staining was carried out using successive washes with ethanol of 50°, 70°
25 and 90°. GUS positive zones were identified as those coloured blue. Light photographs were
26 made with a dissecting microscope (MZ28 Leica, www.leica-microsystems.com/) and a bright-
27 field microscope (Diaphot Nikon, <http://www.nikon.com>).
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34 **Results**

35 *PsPMEP isolation and sequence analyses*

36 In order to isolate and characterize pollen specific genes belonging to the PMEs family in pea,
37 mRNA from pollinated stigmas and pollen was amplified by RT-PCR using degenerated primers
38 of well conserved regions present in different pollen specific PMEs. The RT-PCR fragment
39 obtained was used in the screening of a pea genomic library and five putative clones were
40 isolated. After four consecutive rounds of screening, a purified phage clone containing a full size
41 cDNA was subjected to further analysis. A 4800 pb cDNA fragment was sequenced and, after
42 verifying that contained a gene with homology to PMEs, was named *PsPMEP* (GenBank
43 accession n. KC964536). The coding region is comprised between the nucleotides 2139 (ATG)
44 and 4009 (TGA). This region is interrupted by one intron at position 3183-3269. At nucleotides
45 4813 to 4818 was located a consensus sequence of polyadenylation (AATAAT).
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54 The deduced protein sequence has 584 aminoacids, an estimated molecular weight of
55 65.57 kDa and an estimated isoelectric point of 6.83. The hydropathic profile of *PsPMEP* shows
56 a region of hydrophobic amino acids. This region is located between the amino acids 1 and 29,
57 and could represent the signal peptide. Protein sequence comparison with different PMEs shows
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1 that the mature protein has 353 amino acids, an estimated molecular weight of 40.10 kDa and an
2 estimated isoelectric point of 8.01.
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4 The deduced amino acid sequence of the mature protein shows high homology with other
5 pollen specific PME's of different plant species, including Inverted Repeat-Lacking Clade (IRLC)
6 legumes like *Medicago truncatula* (Q9SC89, Q9SC90) and *Medicago sativa* (Q42920). Other
7 plant species with pollen specific PME's showing homologies with PsPMEP are: *Arabidopsis*
8 *thaliana* (AAX13972, O80722, Q9FJ21, AEE35001, ABI97858.1), *Brassica napus* (P41510),
9 *Brassica rapa* (Q42608), *Petunia integrifolia* subsp. *Inflate* (Q43043), *Nicotiana tabacum*
10 (AAX13972), *Salix gilgiana* (Q9MBB6) and *Zea mays* (Q24596). A Neighbor-Joining Tree
11 regarding the homologies between PsPMEP and the selected pollen specific PME's is shown in
12 Fig. 1. We inferred the phylogeny based on the alignment of mature enzyme sequences of 14
13 representative PME's from pollen, which are clustered into four clades (A to D). The low bootstraps
14 values suggest that sequences comprised in cluster A forms only one clade. All four clades are
15 well supported by bootstrap. Remarkably, several of the lineages, including PsPMEP, show
16 evidence of accelerated rates of evolution. However, to identify signatures of accelerated
17 evolution would require further evolutionary analysis that may shed light on the selective
18 constrains underlying such accelerated evolution.
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28 *PsPMEP structure analysis*

29 The analysis of PsPMEP sequence revealed that it is a typical group 2 pectin methylesterase
30 composed by a pre region (signal peptide), comprising amino acids 1 to 29, a PME I domain,
31 which should have an inhibitory effect on the enzymatic activity, and a catalytic PME domain. A
32 processing signal (RRLL sequence) is present between the two domains (Fig. 2a and b). Only the
33 polypeptide region corresponding to the PME domain is present in the cell wall as mature protein.
34 A search of the protein Data Bank for homologues of PsPMEP yielded carrot PME, with a
35 sequence identity of 46%, as the best available template to model the mature protein. Fig. 2c
36 shows the model of the PME domain. Similarly to other proteins of the CE8 family, the structure
37 consists of a single domain made of right-handed parallel β -strands folded into a triangular prism.
38 Several motifs highly conserved among PME's (underlined amino acids in Fig. 2a and depicted in
39 blue in Fig. 2c) contain essential residues (Q363, Q385, D386, D407 and R474) involved in
40 substrate binding and catalysis. Fig. 2d shows a detail of the catalytic site of the enzyme. The
41 positioning of residues D407 and D386 agree with their presumed role as nucleophile and
42 acid/base catalyst, respectively, on the deesterification reaction.
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53 *PsPMEP expression pattern*

54 Northern blot analysis showed that this gene is specifically expressed in stamens from 4 days
55 before anthesis until anther dehiscence (Fig. 3a). This timeframe corresponds to the
56 developmental stage in which the microspore mitosis is over and the walls (exin and intin) of the
57 pollen grain begin to be synthesized (pollen maturation). No *PsPMEP* expression was located in
58 other vegetative or floral tissues (Fig. 3b and c). At stage 2 days before anthesis, *PsPMEP*
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1 expression was specifically located in anthers and in pollinated carpels after anthesis (Fig. 3c).
2 These results indicated that *PsPMEP* could be a pollen specific gene.
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4 The expression analysis of this gene was also performed by *in situ* hybridization on
5 paraffin sections of pea anthers at different developmental stages. We observed that the gene
6 expression is located only in the pollen grains (Fig. 4a and b). Furthermore, "whole mount"
7 hybridizations using isolated pollen grains during the stage of anthesis indicated gene activity in
8 mature pollen (Fig. 4c and d).
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10 11 12 *Characterization of the PsPMEP promoter*

13 To characterize the *PsPMEP* promoter region, 2138 bp upstream of the transcription start codon
14 were sequenced. The sequence analysis of this region showed the presence of different
15 regulatory motifs involved in the specificity and intensity of pollen gene expression. Three *cis*
16 regulatory elements of special relevance were identified: the AGAAA and AATTGA motifs that
17 have been described to be responsible of the pollen specific expression of different genes and
18 three CArG boxes showing the consensus motif C[AT]₈G, which is preferentially bound by the
19 MADS domain transcriptional regulator AGL15. The well conserved *cis* regulatory element
20 AATTGA, located at positions -647(+), -845(-), contains the TGA triplet that was shown to
21 comprise an active part in other similarly regulated pollen specific promoters. The pollen specific
22 motif AGAAA was located at positions -187, -622, -1037, -1170, -1436(+), -2097(-). The CArG
23 boxes for AGL15 were located at positions -266, -384, -985(+/-). Other putative pollen specific
24 motifs identified in the *PsPMEP* promoter sequence are summarized in supplementary table S2.
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33 The functional analysis of the *PsPMEP* promoter was achieved by genetic transformation
34 of *Arabidopsis thaliana* plants, using a chimaeric construct in the vector pBI101 with the *PsPMEP*
35 promoter driving the expression of the *uidA* reporter gene (Fig. 5a). The results obtained showed
36 a similar expression pattern of this gene in *A. thaliana* when compared with the pollen specific
37 expression of *PsPMEP* in pea. GUS activity was located in anthers (pollen sacs) from 4 days
38 before anthesis to anthesis (Fig. 5c and d) and it was restricted to the later developmental stages
39 of the pollen grains (pollen maturation; Fig. 5d, arrow). High GUS expression was also detected
40 during pollen germination (Figure 5e, arrow). No GUS activity was detected in leaves (Fig. 5f),
41 shoots (Fig. 5g), roots (Fig. 5h), unpollinated carpels (Fig. 5i), siliques (Fig. 5j) or seeds (Fig. 5k).
42 GUS expression was maintained during pollen tube elongation along the transmitting tract of the
43 style and ovary (Fig. 5l, arrow). This expression pattern was maintained till the pollen tube
44 reached the embryo sac in the ovule (Fig. 5m, n; arrows). These results indicated that the 2138
45 bp fragment contains the regulatory sequences necessary to direct the *PsPMEP* expression,
46 showing the same pollen specific pattern in *Arabidopsis* than in pea. In addition, we detected that
47 *PsPMEP* is also expressed during pollen germination and pollen tube growth.
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Discussion

Pollen tube growth and progression in the carpel is an important step in the sexual reproduction of a flowering plant. Progression of the pollen tube through stigma, style, and transmitting tract is essential for delivery of sperm nuclei to the egg cells embedded deeply within female tissues. During this process the pollen tubes invade the stigmatic tissue, penetrate the style, and deposit the two sperm cells into the embryo sac where they fuse with the egg and central cell to form the zygote and endosperm. However, the genetic and molecular mechanisms that control the penetration of pollen tubes through stigmatic and stylar tissues are currently poorly understood. The wall of the pollen tube tip is composed of a single layer of pectin and, unlike most other plant cell walls, does not contain cellulose or callose. Pectin methylesterases (PMEs) likely play a central role in the pollen tube elongation and determination of pollen morphology (Tian et al. 2006). Moreover, a compatible interaction between pollen tubes and stigmatic cells is required for triggering degradation of the stigmatic and stylar cell walls (Atkinson et al. 1993; Hiscock et al. 1994; Johnson and Preuss 2002; Lord and Russell 2002).

PME-related genes are expressed in pollen of many diverse plant species, including legumes like alfalfa (Qiu and EricKson 1995) or *Medicago truncatula* (Rodríguez-Llorente et al. 2004). Here we described a pea pollen specific gene that codifies a protein with homology to PMEs and with a putative role in pollen maturation, pollen germination and pollen tube growth. PsPMEP sequence analysis showed the typical composition of group 2 methylesterases (Pelloux et al. 2007) made of a pre-region (signal peptide), which will drive the protein to the endoplasmatic reticulum, a pro-region (PMI, inhibitory domain) present in many plant PMEs and invertases and a catalytic domain (PME, mature protein). The pro-PME is modified in the Golgi and secreted later to the apoplastic space. Only the region located between the amino acids 260 to 560 of PsPMEP would be finally present in the cell wall as mature protein. This catalytic domain, presumably active, is highly similar to carrot PME (Johanson et al. 2002), which we used as template for modelling. Markovic and Janecek (2004) carried out a comparison of 127 amino acid sequences of the CE8 family. Their analysis showed the existence of five conserved motifs: 44_GxYxE, 113_QAVAL, 135_QDTL, 157_DFIFG, 223_LGRPW (*Daucus carota* numbering) holding critical residues for substrate binding and enzyme activity. Thus, Q113 and Q135 form an anion hole which stabilizes the negatively charged transition product of the catalytic reaction. D157 and D136 are respectively the nucleophile and acid/base catalyst whereas R225 interacts with the nucleophile assuring its right spatial orientation. All these residues are present and correctly positioned in mature PsPMEP, strongly supporting that *PsPMEP* encodes for a functional esterase.

In the *PsPMEP* promoter region we have identified a conserved *cis* regulatory element (AATTGA) at position -647/-642 upstream of the ATG codon, which has been described to be responsible of the pollen specific expression in the alfalfa *MsPG3* promoter (Rodríguez-Llorente et al. 2004). Moreover, analysis of the *MsPG11* regulatory region revealed the presence of an AAATGA motif, which was described by Weterings et al. (1995) in the *NTP303* promoter of *N.*

1 *tabacum* and the *Bp10* promoter of *B. napus* as a putative pollen specific cis-regulatory element.
2 In all these motifs, the TGA triplet was shown to be important for the pollen specific activity. The
3 possibility that expression of the reporter gene in the pollen could be controlled through this
4 AATTGA motif in the *MsPG3* promoter was investigated in *Nicotiana tabacum* plants by mutating
5 the TGA triplet. This TGA sequence was changed to AAA and the resulting mutated version of
6 the complete *MsPG3* promoter was fused to the *uidA* (GUS) reporter gene and introduced into
7 tobacco. A low level of expression of the reporter gene in the transgenic plants carrying the
8 mutated promoter region suggested that the expression of this promoter in tobacco pollen grains
9 was effectively regulated through the AATTGA element. Other interesting motif present in the
10 *PsPMEP* promoter is AGAAA, which is responsible of the specific activation in pollen of the
11 tomato *LAT52* gene (Bate and Twell 1998).

12 We have also identified three CArG boxes showing the consensus motif C[A/T]₆G, which
13 is preferentially bound by the MADS domain transcriptional regulator AGAMOUS-Like 15 (AGL15;
14 Tang and Perry 2003). MADS-box genes encode a large family of transcription factors, most of
15 them are expressed during reproductive development controlling flowering time, meristem and
16 floral organ identity and fruit development. *AGL15* is the only member of the MIKC subgroup
17 identified to date that is preferentially expressed during embryo development (Zhu and Perry
18 2005). Therefore, *PsPMEP* could be a target gene of the MADS domain protein AGL15. However
19 AGL15 regulatory function at the molecular level is still missing. Therefore, the presence of the
20 above mentioned CArG boxes suggests that *PsPMEP* could be a target gene of the MADS
21 domain protein AGL15. This molecular interaction, if confirmed experimentally, could help to
22 uncover a role for AGL15 in the control of pollen development.

23 In higher plants, different PME isoforms are encoded by multiple gene families (Richard
24 et al. 1996; Micheli et al. 1998; Markovic and Janecek 2004) and act in the demethylesterification
25 of cell wall pectin, contributing to different processes of plant development (Micheli 2001).
26 Therefore, the *PsPMEP* product may be involved in growth of the pollen tube by modification of
27 the pollen tube wall. *PsPMEP* was expressed specifically in developing pollen grains and it is not
28 expressed elsewhere. Functional analysis of the *PsPMEP* promoter fused to the *uidA* gene
29 showed a similar expression pattern of this gene in *A. thaliana* when compared with the pollen
30 specific expression of *PsPMEP* in pea. GUS expression was located initially in the anthers (pollen
31 sacs) at 4 days before anthesis till anther dehiscence and it was restricted to the later
32 developmental stages of the pollen grains (pollen maturation). High GUS expression was also
33 detected during pollen germination and the expression was maintained during pollen tube
34 elongation along the transmitting tract of the style and ovary. This expression pattern was
35 maintained till the pollen tube reached the embryo sac in the ovule. These results indicated that
36 the *PsPMEP* promoter is also functional in a non-leguminous plant, showing the same pollen
37 specific expression pattern than in pea.

38 Studies have shown that mature PMEs could have different modes of action. They may
39 act either randomly or linearly along the pectin chain (Markovic and Kohn 1984; Micheli 2001). It
40 is commonly believed that random demethylesterification of pectin depends on acidic PMEs,
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1 whereas linear demethylesterification of pectin requires basic PME (Micheli 2001). The PsPMEP
2 mature protein showed a predicted basic isoelectric point of 8.61. Therefore, PsPMEP may act by
3 linear demethylesterification of pollen wall pectin. The linear demethylesterification on
4 homogalacturonans by PME gives rise to blocks of free carboxyl groups that could interact with
5 Ca^{2+} , creating a pectate gel (Goldberg 1996). The Ca^{2+} pectate gel is believed to contribute to cell
6 wall stiffening and cell attachment by limitation of the action of endopolygalaturonases and
7 formation of Ca^{2+} pectate gel lawn.
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11 To date there is not any annotation in the GenBank concerning genes with homology to
12 pollen specific PMEs in pea (*Pisum sativum* L.), a model legume also relevant by its agronomic
13 interest. We have identified a new pea pollen specific PME (PsPMEP) and characterized its
14 native expression pattern by Northern-blot and *in situ* hybridization. Also, we introduced a
15 *PsPMEP* promoter-gus gene fusion into *Arabidopsis thaliana* plants, corroborating the spatial and
16 temporal expression of *PsPMEP* previously observed in pea. Our results suggest that *PsPMEP*
17 could be implicated in pollen development and pollen tube growth through stigma, style and
18 transmitting tract within female tissues. Further functional analyses, using reverse genetics
19 approaches, are needed to confirm the role of the PsPMEP protein in such processes. A
20 possibility is to silence the orthologous gene in the model legume *Medicago truncatula* which is
21 relatively easy to transform. In addition, the *PsPMEP* pollen specific promoter seem to be a
22 promising candidate to target expression of a desired gene during late pollen development or
23 pollen tube growth and to induce male sterility, fused to a cytotoxic gene, without affecting plant
24 development (Roque et al. 2007; Pistón et al. 2008; García-Sogo et al. 2010, 2012). This
25 approach could help in overcoming problems related to transgene escape from GM crops to
26 organic or traditional ones.
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38
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44 this special issue.
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49 **Figure legends**

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52 **Fig. 1. Neighbor-Joining Tree of selected pollen specific PMEs.** The numbers next to the
53 nodes refer to bootstrap values from 1000 pseudo-replicates. Accession numbers for the PME
54 proteins used in this N-J tree are: AAX13972, O80722, Q9FJ21, AEE35001 and ABI97858.1
55 (*Arabidopsis thaliana*); P41510 (*Brassica napus*); Q42608 (*Brassica rapa*); Q43043 (*Petunia*
56 *integrifolia* subsp. *inflata*); AAX13972 (*Nicotiana tabacum*); Q9MBB6 (*Salix gilgiana*); Q24596
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1 (Zea mays); Q9SC89, Q9SC90 (*Medicago truncatula*); Q42920 (*Medicago sativa*) and
2 KC964536, PsPMEP (*Pisum sativum*). Branch lengths are proportional to sequence divergence.
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5 **Fig. 2. Structure of PsPMEP.** a. Deduced amino acid sequence of the cDNA-encoded
6 polypeptide. The predicted signal peptide and the processing motif (RRLL) are represented in
7 bold type. The PMI domain is marked in red. The sequence corresponding to the PME domain is
8 shadowed. Blocks of residues highly conserved among pectin methylesterases are underlined.
9 Putative catalytic residues, D386 (red) and D407 (blue) are marked. b. Schematic representation
10 of the domain structure of PsPMEP. c. Homology-based model of the pectin methylesterase
11 domain. Groups of highly conserved amino acids are depicted in blue color. d. Detail of the pectin
12 methylesterase active site showing the putative catalytic residues D386 (acid-base catalyst) and
13 D407 (nucleophile).
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20 **Fig. 3. Northern blot analysis of the PsPMEP expression in different vegetative and floral**
21 **tissues of pea.** a. Expression was detected in stamens (St) from 4 days before anthesis till
22 anthesis, but not in floral buds (F) at 6-8 days before anthesis. b. No expression was detected in
23 vegetative tissues like roots (R), mature shoots (S), young shoots (YS), leaves (L) and tendrils
24 (T). c. Anther-specific expression of *PsPMEP* in flowers at 2 days before anthesis and in
25 pollinated carpels after anthesis (PC). There is no expression in the other floral organs: sepals (S-
26 2), petals (P-2) and unpollinated carpels (C-2) at 2 days before anthesis.
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31 **Fig. 4. PsPMEP expression pattern analyzed by in situ hybridization.** *In situ* hybridization on
32 paraffin sections of pea anthers at 2 days before anthesis. a. Control section hybridized with a
33 *PsPEMP* sense probe. b. Anther section hybridized with the antisense probe. *PsPMEP*
34 expression was located exclusively in the pollen grains and not in other anther tissues. c. "Whole
35 mount" of isolated pollen grains hybridized with the sense probe as control. d. "Whole mount"
36 hybridization with the antisense probe on isolated pollen grains at anthesis showing *PsPMEP*
37 expression. Co: Connetive tissue; Ep: epidermis; Po: pollen grains; En: endotecium; Vb: vascular
38 bundles.
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45 **Fig. 5. Expression analysis of the PsPMEP promoter fused to the uidA gene in transgenic**
46 **Arabidopsis thaliana plants.** a. *PsPMEP::uidA* chimaeric construct in the vector pBI101
47 containing the nopaline synthase constitutive promoter (nos-pro) fused to the *nptII* gene
48 (kanamycin resistance), the *PsPMEP* promoter fused to the *uidA* gene (β -glucuronidase, GUS-
49 intron) and the polyadenylation signal of the nopaline synthase gene (nos-ter) as terminator at the
50 3' ends of both genes. b. Transgenic *A. thaliana* flowers at one day before anthesis transformed
51 with the empty vector pBI101 (control). c. Transgenic *A. thaliana* flowers carrying the chimaeric
52 construct *PsPMEP::uidA* showing GUS expression in the pollen sacs of developed anthers. d. Id.
53 a detailed view of the pollen sacs (arrow) with mature pollen grains showing GUS expression. e.
54 GUS expression in the germinating pollen grains (arrow) located on the stigmatic papillae. f. No
55 GUS expression in leaves. g. Id. in shoots. h. Id. in roots. i. Id. in unpollinated carpels. j. Id. in
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1 siliques. **k.** Id. in seeds. **I.** GUS expression in the elongated pollen tubes along the stylar and
2 ovarian transmitting tract. **m-n.** The *PsPMEP* expression pattern was maintained till the pollen
3 tube (Pt) reached the embryo sac (Es) in the ovule (Ov).
4

5 6 **Supplementary material**

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9 **Table S1.** Primers used to sequence the *PsPMEP* genomic clone.

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11 **Table S2.** Putative pollen specific motifs identified in the *PsPMEP* promoter region (-1 to -2133).
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Figure 1
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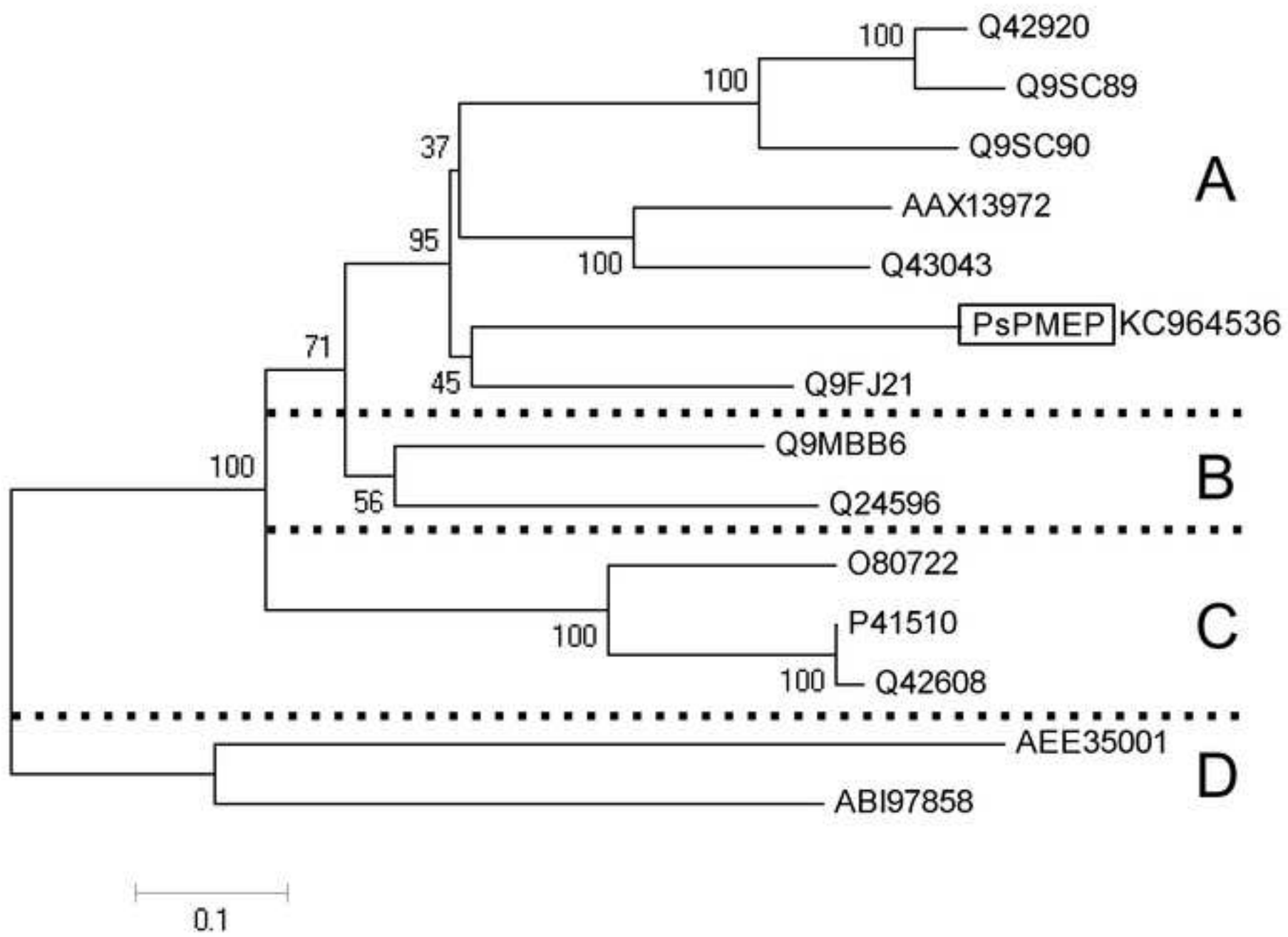


Figure 2

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QTICQPTDYKKECVDTLTAEAEAGNVTPKELIKIAFNVTMKKIGEQIKATDLMHEVEKD 120
PRSKDAIDTCKQLMDLSIGEFARAMDGIHQFDLKDIDRI LMNLKVLNGAVTYMDTCVDG 180
FQNTTTDAGKKMKDILTS SMHSSNALAI ISDFADTVTSWNVTKIFGRRLLEDTPSWVS 240
EHRMLLDAKTSSFKLKPNTVALDGTGDVKTINEALLKVPEKSEKPF FFIYIKEGVYNEYV 300
EVGKHMTYVVFELGDGGQKSRIITGSKNFIDGVNTYKTASVAIQGDHFTA INMGFENSAGAH 360
KHQAVLRVQGDKSIFFNC SMDGYQDTLYVHTMRQFYRDCTISGTI FIFGNALSVFQNC 420
KFVVRKPMANQQCI VTAQGRKERFQPSAIVIQGSIVADPEFYPVRFDHKAYLARPWKNF 480
SRTIFMDTFIDDLIHPDG YMPWQTP EGFSGMDTCFYAEYHNYGPGSDKSKRVHWAGIWSL 540
NSKAARWFAPS KFFHGS DWIEETGV PFFSSIPDHRHKKTVLTW 584

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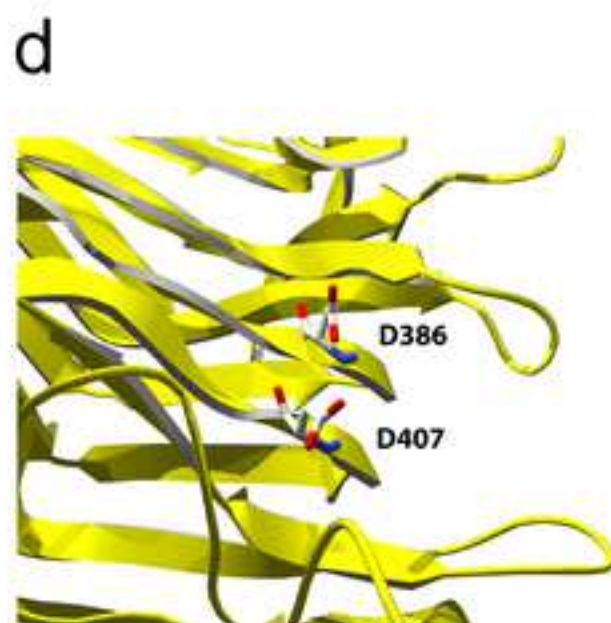
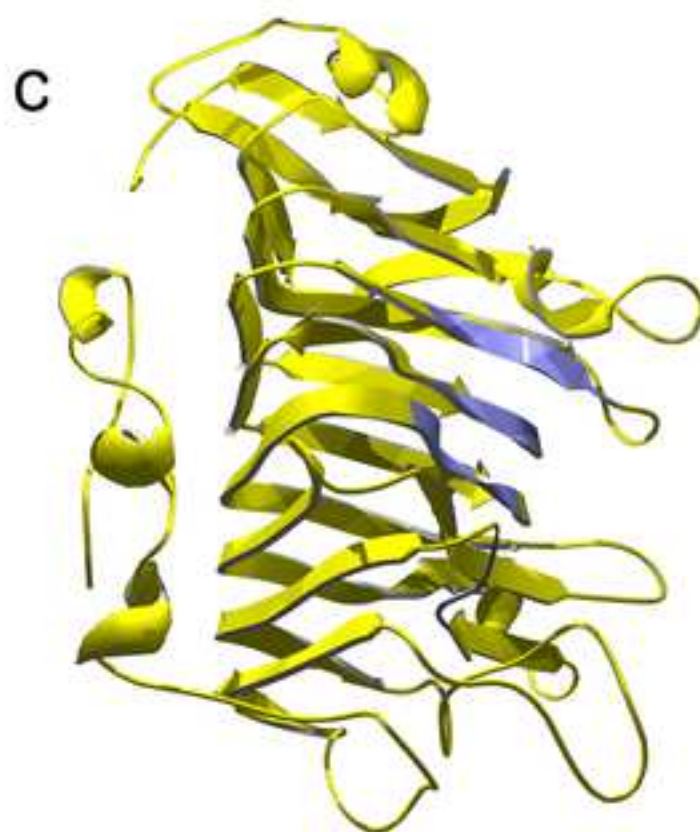
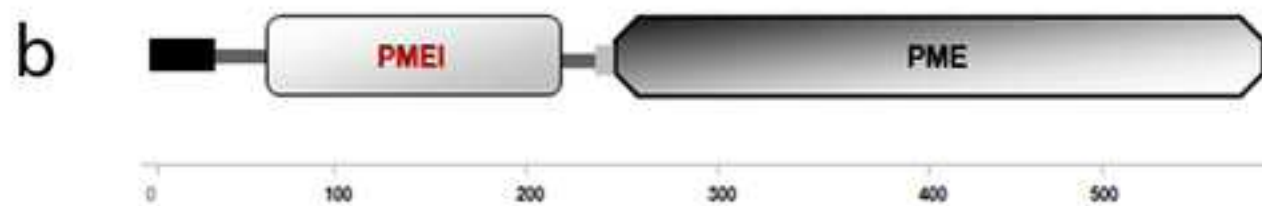


Figure 3

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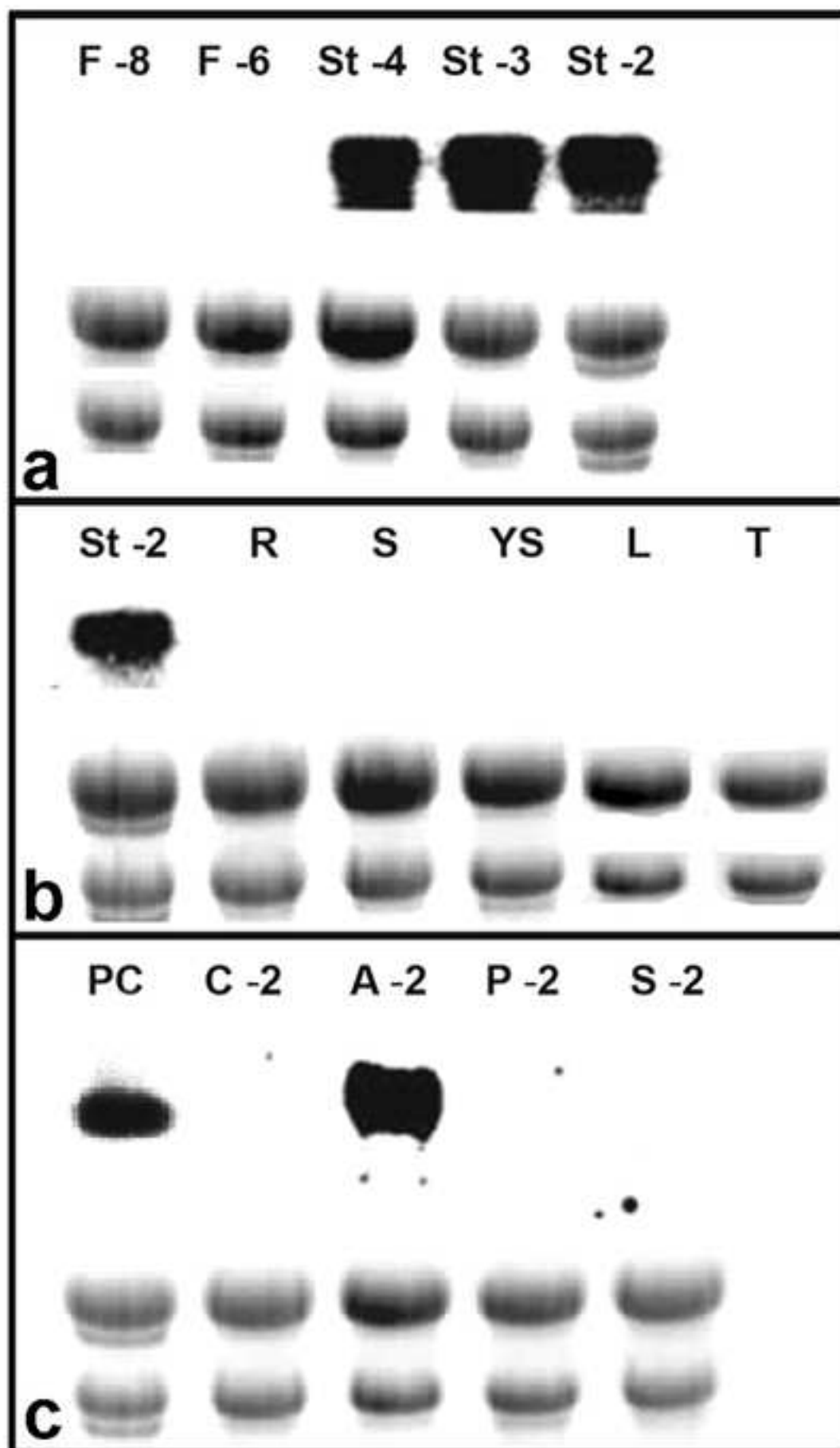


Figure 4

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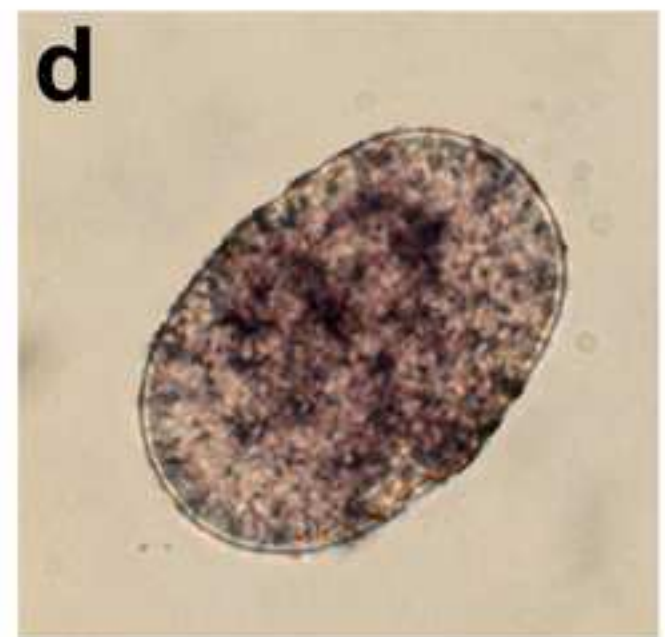
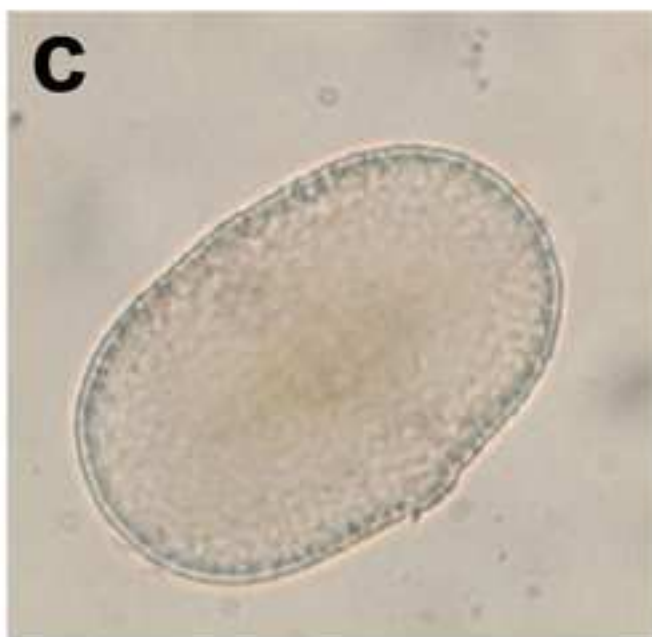
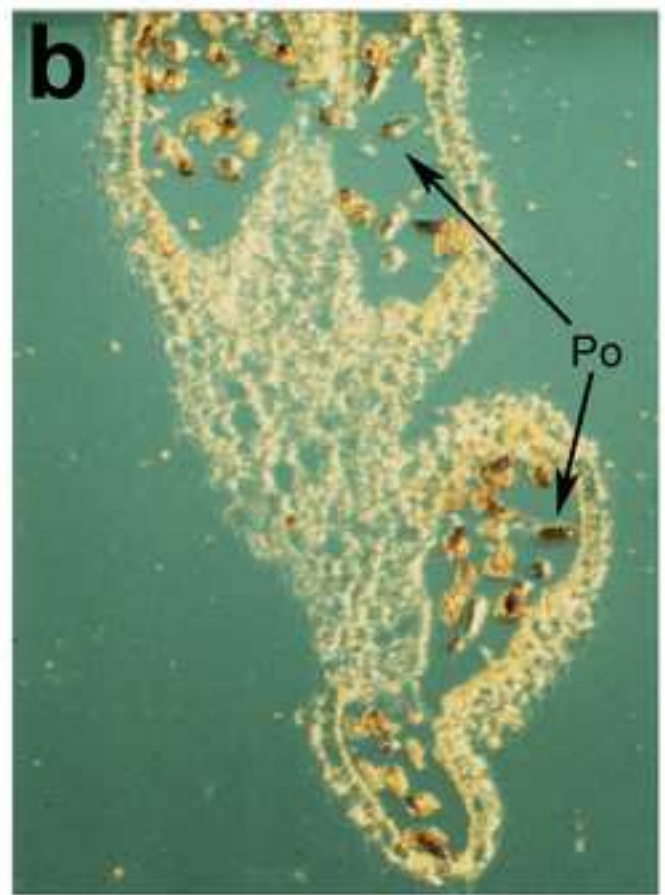
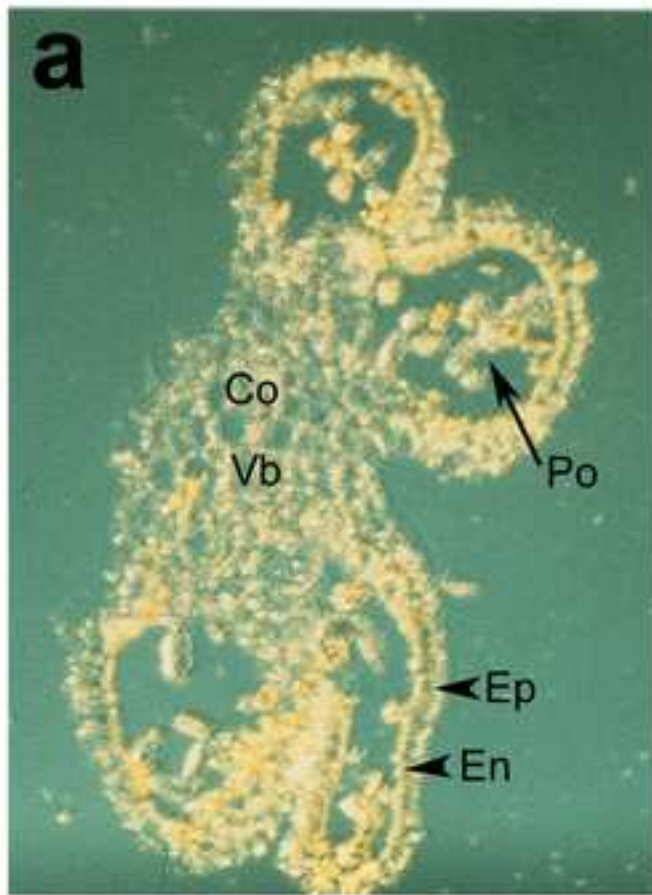


Figure 5
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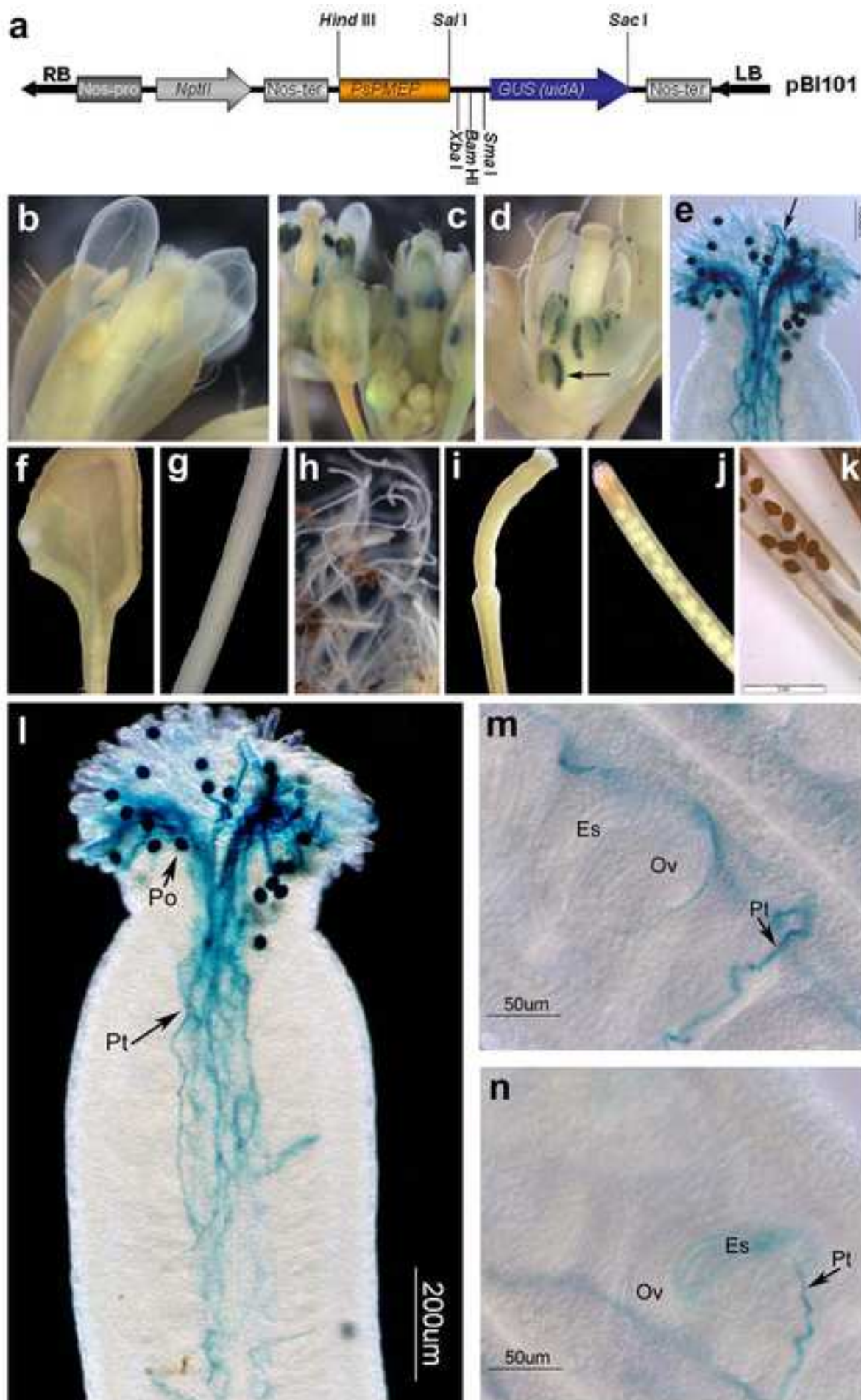


Table S1.

Primers	Sequence (5' to 3')
PEC2	GCCCACAAAACATCAAGCCCGTGG
PEC3	CTTACATGGTGATTTTCGATAGTGTG
PEC4	GTGTGGCTTCCGTGTTTTACTATTTGTAG
PEC5	GGTGATGGTGGCCAGAAATCAAG
PEC6	CTTTCCATTGGAGAATTCGCCCGAG
PEC8	GGCAGAGGGTGGTGATGC
PEC7	GAATATATCTTCTCAAAGATAAATA
PEC1R	CTGACCCATGAAGGTGTATCG
PEC2R	CTGATTCCAATGCAAAATAGCTTATGG
PEC3R	CGCATGGTGTGTACGTAAAG
PEC4R	CACCATGTAAGCACTGTCTTT
PROPEC1	CCTCACAAGCCAATTATCTCTTC
PROPEC2	GAAGAGATAATTGGCTTGTGAGG
PROPEC3	CTGAAATGATGGCGAGAGCATTGC
PROPEC3B	GTATATGTGGTTTATTGGTTGAGG
PROPEC4B	GATGCCTATATACTTGATGCC

Table S2

Promoter	Position	Consensus	Reference
CArG-box (<i>AGL15</i>)	-266, -384, -985 (+/-)	C(AT) ₈ G	Tang & Perry, 2003
<i>LAT52</i>	-187, -622, -1037, -1170, -1436 (+), -2097 (-)	AGAAA	Bate & Twell, 1998
<i>LAT52</i>	-846 (+)	GTCAATT	Twell et al., 1989, 1990, 1991
<i>TUA1</i>	-213, -880, -1004 (+), -164, -723, -1198, -1947 (-)	TGTGG (PB motif)	Carpenter et al., 1992
<i>AVP1</i>	-243, -611, -992, -1342, -1605 (+)	AGTCA (similar to "Q element")	Mitsuda et al., 2001
<i>ZM13</i>	-1547 (+), -1469 (-)	"Q element" AGGTCA	Hamilton et al., 1998
<i>ZM13</i>	-223 (-)	Sequence TGGTTA of the 52/56 motif	Hamilton et al., 1998
<i>Bra r 1</i>	-148, -864, -935, -947, -1052, -1150, -1280, -1447, -1516, -1522, -2008 (+), -61, -102, -1067, -1319, -1337, -1644, -2103 (-)	Sequence GTGA of the 56/59 motif	Okada et al., 1999
<i>MsPG3</i>	-647 (+), -845 (-)	AATTGA	Weterings et al., 1995