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Comprehensive expression profiling of the pectin methylesterase gene family during silique development in *Arabidopsis thaliana*

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Abstract Pectin methylesterases (PME, EC. 3.1.1.11) are enzymes that demethylesterify plant cell wall pectins in muro. In *Arabidopsis thaliana*, putative PME proteins are thought to be encoded by a 66-member gene family. This study used real-time RT-PCR to gain an overview of the expression of the entire family at eight silique developmental stages, in flower buds and in vegetative tissue in the *Arabidopsis*. Only 15% of the *PMEs* were not expressed at any of the developmental stages studied. Among expressed *PMEs*, expression data could be clustered into five distinct groups: 19 *PMEs* highly or uniquely expressed in floral buds, 4 *PMEs* uniquely expressed at mid-silique developmental stages, 16 *PMEs* highly or uniquely expressed in silique at late developmental stages, 16 *PMEs* mostly ubiquitously expressed, and 1 *PME* with a specific expression pattern, i.e. not expressed during early silique development. Comparison of expression and phylogenetic profiles showed that, within phylogenetic group 2, all but one *PME* belong to

the floral bud expression group. Similar results were shown for a subset of one of the phylogenetic group, which differed from others by containing most of the *PMEs* that do not possess any PRO part next to their catalytic part. Expression data were confirmed by two promoter:GUS transgenic plant analysis revealing a *PME* expressed in pollen and one in young seeds. Our results highlight the high diversity of PME expression profiles. They are discussed with regard to the role of *PMEs* in fruit development and cell growth.

Keywords *Arabidopsis* · Cell wall · Pectin methylesterase · Silique · Real-time RT-PCR

Abbreviations PME: Pectin methylesterase · RT-PCR: Reverse transcription polymerase chain reaction · DAF: Days after flowering

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Introduction

The plant cell wall is a highly complex structure consisting of cellulose, hemicellulose, pectins and proteins, which provide cell shape and modulate growth. Among these polysaccharides, pectins represent nearly 35% of the total dry weight in dicotyledonous cell walls (Cosgrove 2001). Pectins, which consist of a backbone of homogalacturonan and rhamnogalacturonan, are polymerised in the *cis* Golgi apparatus. Homogalacturonans are subsequently methylesterified in the medial Golgi apparatus by pectin methyltransferases (PMT). The addition of side chains to the backbones gives rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan. It occurs in the trans Golgi apparatus, before exocytosis of pectins to the cell wall where they contribute to the structuration of the middle lamella and the primary cell wall. Pectin synthesis and metabolism is a series of complex enzymatic processes where PME plays a central role. *PMEs* (EC. 3.1.1.11) are enzymes belonging to the class 8 of the carbohydrate esterases (CAZY:

<http://www.afmb.cnrs-mrs.fr/CAZY/> and Coutinho et al. 2003). They are involved in the demethylesterification of pectins in muro, creating carboxyl groups which play a role in the complexation of calcium ions, increasing cell wall firmness (Willats et al. 2001). In addition, action of PME is required prior to the action of polygalacturonases (PG), which are involved in the degradation of the pectic matrix during fruit ripening (Wakabayashi et al. 2003). Thus, PMEs contribute both to the firming and softening of the cell wall.

The crystalline structure of a plant PME has been determined and compared with bacterial PMEs, which allowed the identification of conserved amino acids residues (Johansson et al. 2002). On this basis, in the Arabidopsis genome, 66 genes have been suggested to potentially encode pectin methylesterases. PME sequences consist of two types: the type I, which has a long N-terminal PRO region (pre-pro-proteins) and the type II which has a short or absent PRO region (Micheli 2001). Several functions for the PRO region have been suggested, including targeting of PME to the cell wall, correct folding of the enzyme and inhibition of the PME activity (Micheli 2001). Only the mature part of the protein can be extracted from the cell wall, but the exact mechanism of the PRO-mature cleavage remains unknown. Numerous studies (see Giovane et al. 2004 for review) have shown that the PRO region shows similarities with PME inhibitors (PMEI), and it was recently demonstrated that the PRO region plays a role in preventing an early demethylesterification of pectins in the Golgi apparatus (Bosch et al. 2005).

So far, very few PME sequences have been shown to code proteins biochemically active as carbohydrate esterases. The high number of putative PME sequences in the Arabidopsis genome suggests that these proteins could be involved in different developmental processes and/or act on different classes of pectins. PMEs have been notably shown to play a role in such diverse processes as cambial cell differentiation (Micheli et al. 2000), hypocotyl elongation (Al-Qsous et al. 2004), microsporogenesis (Lacoux et al. 2003) and as host receptor for the tobacco mosaic virus movement protein (Chen and Citovsky 2003). Recently, VANGUARD1, encoding a pectin methylesterase homologous gene, has been shown to be specifically localised in Arabidopsis pollen grains and to play a central role in pollen tube growth in the style (Jiang et al. 2005). NtPPME1 was shown to have the same function in tobacco (Bosch et al. 2005). In *Medicago sativa* and *Medicago truncatula*, eight PME genes have been isolated, two of them showing a flower-specific expression pattern, while another one was shown to be expressed in roots during the nodulation process (Rodriguez-Llorente et al. 2004). Among all developmental processes studied so far, the main highlight has been put on the implication of PME in fruit development. PME have notably been shown to be involved in fruit maturation in tomato (Eriksson et al. 2004), *Phaseolus* (Stolle-Smits et al. 1999), strawberries (Castillejo et al. 2004), berries (Barnavon et al.

2001), bananas (Nguyen et al. 2002). The results show either an increase or a decrease in PME activity during fruit maturation.

Silique formation in Arabidopsis is a complex process where the embryo and post-fertilization carpel development occur in parallel for up to 20 days. At the embryo level, development involves three distinct phases: (1) early morphogenesis, including division and segmentation of the zygote (up to 7 days after flowering, DAF); (2) maturation phase, which includes storage of lipids (up to 17 DAF) and (3) late maturation phase (up to 20 DAF) which mainly corresponds to the acquisition to desiccation (Baud et al. 2002). After ovule fertilization, cells of the carpel undergo division, expansion and differentiation to form the various tissues of the mature silique: endocarp, mesocarp and exocarp. Longitudinal growth is notably due to cell expansion in all layers of the carpel, while final silique maturation involves the formation of the dehiscence zone (DZ), a non-lignified separation layer placed between lignified cells, which will allow seeds dispersal (Ferrandiz 2002). Therefore, Arabidopsis silique development requires major changes in plant cell wall composition, structure and hydration level. Proteins involved in those processes, such as PMEs, are likely to undergo drastic variation in the expression of their genes and/or activity levels.

A wealth of public microarray data are available for some silique developmental stages. However, only 60 out of the 66 PMEs are present on the Affymetrix ATH1 genome array, and it was shown that some discrepancies could exist between microarrays and real-time RT-PCR analysis of gene expression (Czechowski et al. 2004). The latter study notably revealed, using real-time RT-PCR, expression specificity between members of conserved gene families unseen in microarray data.

This has prompted us to use real-time RT-PCR to study the relative expression of the 66 genes encoding putative PMEs throughout eight silique developmental stages. For two of the PMEs, RT-PCR results were confirmed using promoter-GUS fusions showing the localisation of expression. The results are discussed with regard to a phylogenetic analysis and compared to freely available microarray data.

Material and methods

Phylogenetic analysis

Sixty-six putative Arabidopsis PME protein sequences were retrieved from the CAZY website (http://www.afmb.cnrs-mrs.fr/CAZY/CE_8.html) and aligned using ClustalX (Thompson et al. 1997). Phylogenetic tree was drawn with the neighbour-joining method using MEGA software (molecular evolutionary genetics analysis, Kumar et al. 2001) using pairwise deletion; 1,000 replicates were used for bootstrap analysis and the cut-off value was 50%.

Plant material and growth conditions

Arabidopsis thaliana (Col-0, obtained from the Nottingham Arabidopsis Stock Center, Nottingham, UK) wild-type plants were grown on soil in greenhouse under a 16-h photoperiod and day/night temperature of 22°C/20°C. To follow silique development, newly opened flowers were labelled daily for 20 days on 80 plants. The material (vegetative tissue, flower buds and siliques at various development stages) was harvested and frozen under liquid nitrogen before storage at -80°C. For young siliques, floral parts were dissected. Sampling was replicated once.

RNA extraction and cDNA synthesis

Tissues were ground in liquid nitrogen and after a preliminary polyphenol and polysaccharide precipitation (Gehrig et al. 2000), RNA was extracted from supernatant using a hot phenol purification protocol (Verwoerd et al. 1989). Total RNA was treated with amplification-grade DNase I (Invitrogen). Absence of genomic DNA contaminants was subsequently confirmed by PCR using primers spanning intronic sequences. RNA integrity was assessed by loading 1 µg of RNA on a 1.2% agarose gel, and RT reactions were performed using the SuperScriptII reverse transcriptase (Invitrogen) following the manufacturer's instructions. Efficiency of RT was assessed by using control genes encoding *ACTIN 2-7-8*, to obtain Ct in the range of 22–23. The *PME* expression study was realised on these cDNAs.

Primer design

Sixty-six putative *PME* nucleotide sequences (excluding pseudogenes), retrieved from the CAZY web server (http://www.afmb.cnrs-mrs.fr/CAZY/CE_8.html), were used to design primers flanking, when possible, an intronic sequence using the LC Probe Design[®] software (Roche; Basel, Switzerland). Primers were designed to obtain an amplified product in a range of 200–500 bp, and their specificity was tested using BLASTn searches (<http://www.ncbi.nlm.nih.gov/BLAST/>). The specificity of the primers was subsequently tested by real-time PCR using increasing dilution of the template. For all primers, the efficiency of the PCR was in the range of 90–100%. The primer sequences are given in Supplementary material I

Real-time PCR conditions and data analysis

Real-time PCR was performed on a Roche LightCycler[®] using the FastStart DNA Master^{PLUS} SYBR Green I kit

(Roche) according to the manufacturer's protocol. The cDNA pool was diluted to a concentration of 5 ng/µl of which 5 µl were used, together with 0.25 mM of specific primers, in a final volume of 20 µl. The following thermal profile was used for all PCR: 95°C for 10 min, 45 cycles of 95°C for 10 s, 64°C for 15 s and 72°C for 15 s. This PCR reaction was followed by a melting curve analysis from 48 to 95°C in 470 s. Crossing threshold (Ct) values, which are the PCR cycle numbers at which the accumulated fluorescent signal in each reaction crosses a threshold above background, were obtained with the LightCycler[®] software 3.5 (Roche) using the second derivative maximum method. Ct values are a function of the amplification efficiency of the respective PCR. *PME* expression was calculated using the RelQuant[®] software (Roche), as a relative expression, for a set 100 bp fragment; compared to the internal control *ACTIN 2-7-8* (Schenk et al. 2003). For this purpose, the ΔCt ($\Delta Ct = Ct(PME) - Ct(ACTIN 2-7-8)$) was used in the following mathematic formula $[PME] = 2^{-\Delta Ct}$ and was corrected by the primer efficiency, based on relative standard curves describing the PCR efficiencies of each target and reference genes. The quantifications were realised on separate cDNAs pools coming from two independent plant-sampling experiments. Data presented correspond to one of the sampling (S1), but results, checked for ten *PMEs*, were similar for the second experiment (S2 = 1.40 × S1, $R^2 = 0.956$; Supplementary material 2). Data analysis and hierarchical clustering were performed according to EPClust (<http://www.ep.ebi.ac.uk/EP/EPC-LUST/>) using the correlation measure-based distance and the average linkage (average distance, UPGMA) clustering method.

Analysis of promoter activity

The promoters of *At1g69940* and *At5g49180* were amplified from genomic DNA using AmpliTaq polymerase (Applied Biosystems) using gene-specific primers pairs:

PCR products were cloned into the pGEM-T Easy vector (Promega), sequenced and subcloned into the binary vector pBI101.3 upstream of the *GUS* coding sequence. Plant transformation, using *Agrobacterium tumefaciens* strain LBA4404, was according to the floral dip method (Clough and Bent 1998). Transformants were selected on kanamycin at 80 µg/ml. *GUS* staining was performed using 0.3 mg/ml X-Gluc in 50 mM phosphate buffer (pH 7) containing 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide. Tissues were incubated for 14 h at 37°C, subsequently destained in ethanol series up to 50%, post-fixed in FAA (50% ethanol, 5% formaldehyde, 10% acetic acid) for 30 min and finally dehydrated completely in ethanol series up to 100%.

Locus	Forward primer	Reverse primer
<i>At1g69940</i>	5'-TCTAGAACTTTTATACATGTTAAATGC-3'	5'GGGATTCCAGTTCACCATCAACCCCAT-3'
<i>At5g49180</i>	5'GGGATTCCAGTTCACCATCAACCCCAT-3'	5'-AAGCTTGGGTAGGATTTATTTCTGTC-3'

Results

Expression of PME_s during silique development

In *Arabidopsis*, silique development, which correlates with seed development, showed a steep increase in length and diameter up to 7–8/9–11 DAF, followed by a sole increase in diameter up to 12–14 DAF (Fig. 1). From 15 DAF, silique starts to dehydrate and prepare for dehiscence, which is concomitant with seed dehydration. This developmental pattern of seed and silique is likely to imply changes in cell wall composition and thereby in the expression and activities of enzymes involved in this process. Indeed, our analysis showed that 85% of all *PMEs* were expressed at one or more stages of development, and that their expression could be clustered into five distinct groups according to their kinetics and level of expression (Fig. 2). These groups were named A to E: Group A consisted of 16 *PMEs* mainly highly or uniquely expressed in silique, particularly during late developmental phases. Group B was restricted to one member, *At1g23200*, which had a very specific profile of expression, at low levels in vegetative and flower stages, not expressed during the 1–2 to 3–4 stages, and expressed at relatively high levels from stages 5–6 onwards. Group C consisted of 16 members, most of them expressed throughout silique development. However, in this latter group, some members (notably *At3g05620*, *At5g04970*, *At5g47500* and *At5g61680*) differed from this general expression pattern. Group D was the largest, having 19 members showing high or unique expression patterns in flower buds. Group E contained 4 *PMEs* whose expression was restricted to early to mid-stage of silique development, and was present neither in vegetative stage nor in flower buds. These results pointed out the high diversity of expression profiles within a gene family together with putative-specific roles for various *PMEs* at a given stage. Moreover, within each group, relative expression levels differed between members to a very high extent (Supplementary material 3). For instance, within group A, at stage 15+, transcript expression varied between 0.021 and 424 for *At5g64640* and *At3g60730*, respec-

tively. In addition, summing the expression of all *PMEs* at a given stage brought some information on the global *PME* expression during silique development, as well as the relative contribution of each *PME* at each stage (Supplementary material 3). The *PME* expression level was relatively low, just above 200, in our vegetative control, which could be related to the physiological stage of the plants onto which the siliques were sampled (growth was close to an end). It is therefore likely that all *PMEs* expressed in young tissues, and in roots, were not taken into account. The total *PME* expression on an overall was very high in flower buds, mainly due to the high expression of several *PMEs* such as *At1g69940*, *At2g47040*, *At3g62170* and *At4g33220*. During silique development, a biphasic *PME* expression pattern was shown with an increase in total expression up to stage 5–6, a drop at stage 7–8 and relatively high levels from stage 9–11 onwards. This would imply that, while specific isoforms could be involved at very specific stage of dry fruit maturation (*At3g60730*, *At5g49180*...), others are ubiquitously expressed throughout silique development and throughout the whole plant (*At4g33220*, *At3g49220*...).

Phylogenetic analysis of the PME family

A phylogenetic tree was built to relate sequences to expression profiles (Fig. 3). The *PME* family clustered in four distinct groups (named group 1 to 4). Group 3 was only composed of two members, *At5g09760* and *At5g64640*. Apart from two sequences (*At1g11370* and *At4g33220*), all the *PMEs* containing a PRO region clustered into groups 1 to 3, whereas *PMEs* which did not contain a PRO sequence were in group 4.

PME expression in floral bud

In order to relate expression patterns and phylogenetic analysis, *PME* expression values are shown for two groups of the phylogenetic tree (Table 1). One of the striking features of this was that, among the phylogenetic group 2 all but one *PMEs* were highly or uniquely expressed in flower bud, and clustered into expression

Fig. 1 Silique developmental stages used for cDNA preparation. Flowers were labelled daily on 80 plants and samples were harvested after 20 days. For stage 1–2, flower parts were dissected. *FB* floral bud, 1–2 to 15+: 1–2 to 15+ days after flowering (DAF)

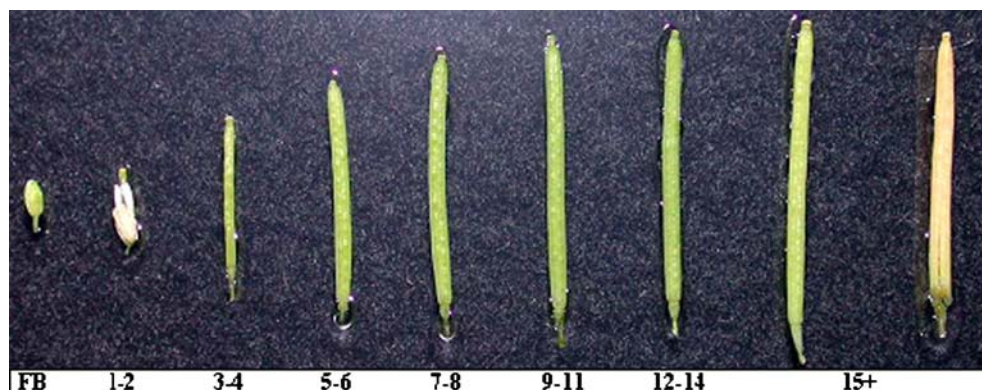
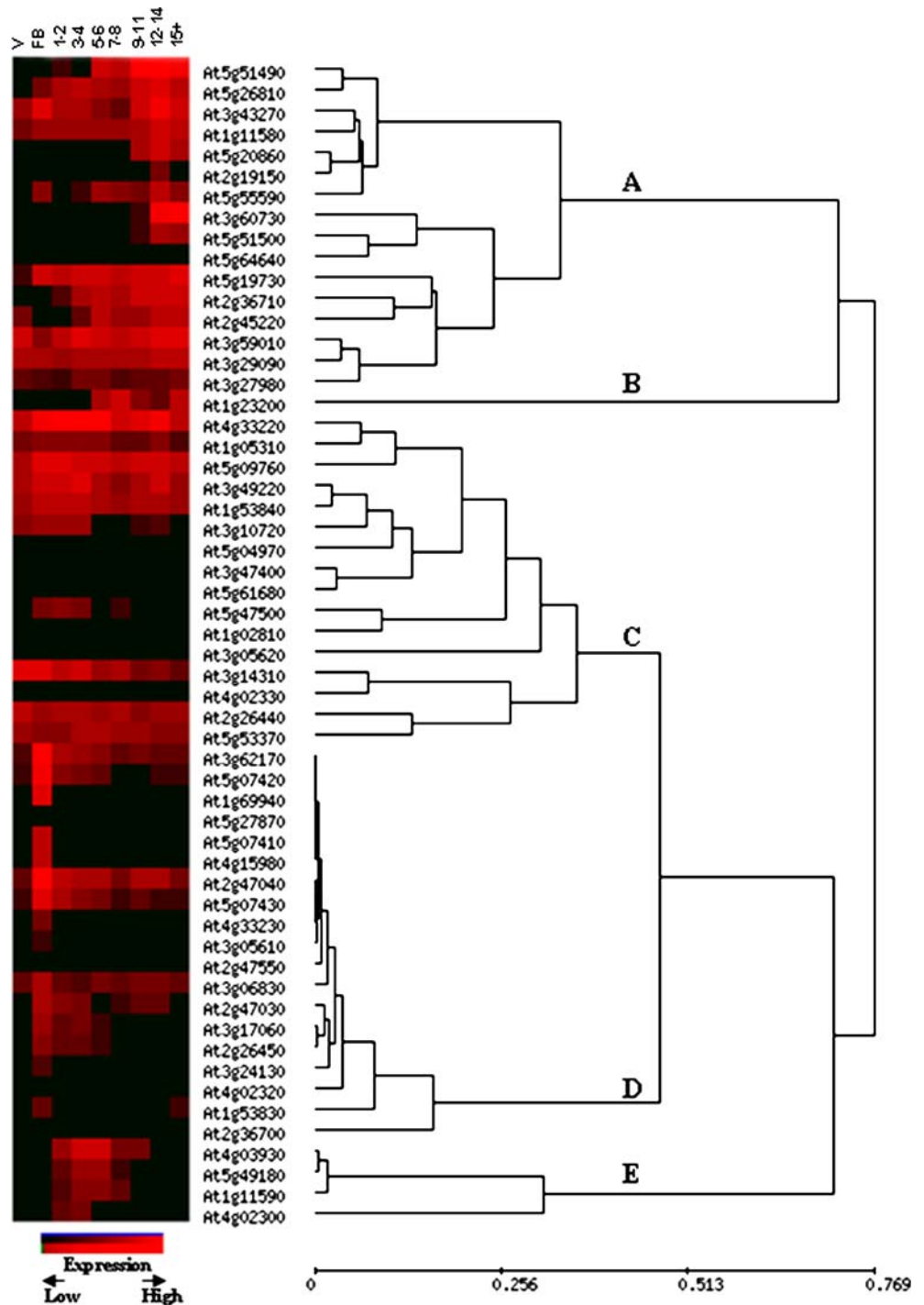


Fig. 2 Expression of PME was studied during silique development by real-time RT-PCR and was quantified relatively to the constitutive control ACTIN 2-7-8. All results are expressed as the ratio $[PME/ACTIN\ 2-7-8] \times 1,000$, and are shown as increasing red colour intensity. The cluster was realised with expression profile data clustering (EPClust) at the European Bioinformatic Institute (<http://www.ep.ebi.ac.uk/EP/EPC-LUST/>) using the data presented in the supplementary material concerning the 56 expressed PMEs. Values represent the relative PME expression as the ratio $[PME/ACTIN\ 2-7-8] \times 1,000$. *V* vegetative control; *FB* floral bud; *1-2 to 15+*: 1-2 to 15+ DAF. The vegetative control used in our study corresponded to leaves (rosettes and caulines) and stems harvested from the same batch of plants as the one used for the sampling of the siliques



group D. Similar results were obtained for a subset (bottom part) of phylogenetic group 4 (Table 1). This points out the high number of *PMEs* expressed in flowers in Arabidopsis. Moreover, our study revealed that *At1g69940*, belonging to expression group D and phylogenetic group 4 (no PRO part), showed an expression pattern restricted to floral buds, at a relatively high level (83.79). In order to localise *At1g69940* promoter activity, Arabidopsis plants were transformed with a

promoter:*GUS* construct. *GUS* staining showed that promoter activity was restricted to mature pollen grains in the anthers and on the stigma (Fig. 4a, b). Moreover, *GUS* staining was visible in pollen tubes within the style (Fig. 4c). Similar results were obtained for two additional *PMEs*, *At5g07410* and *At3g06830* (data not shown). These *PME* isoforms could therefore be involved in pollen maturation and/or pollen tube growth.

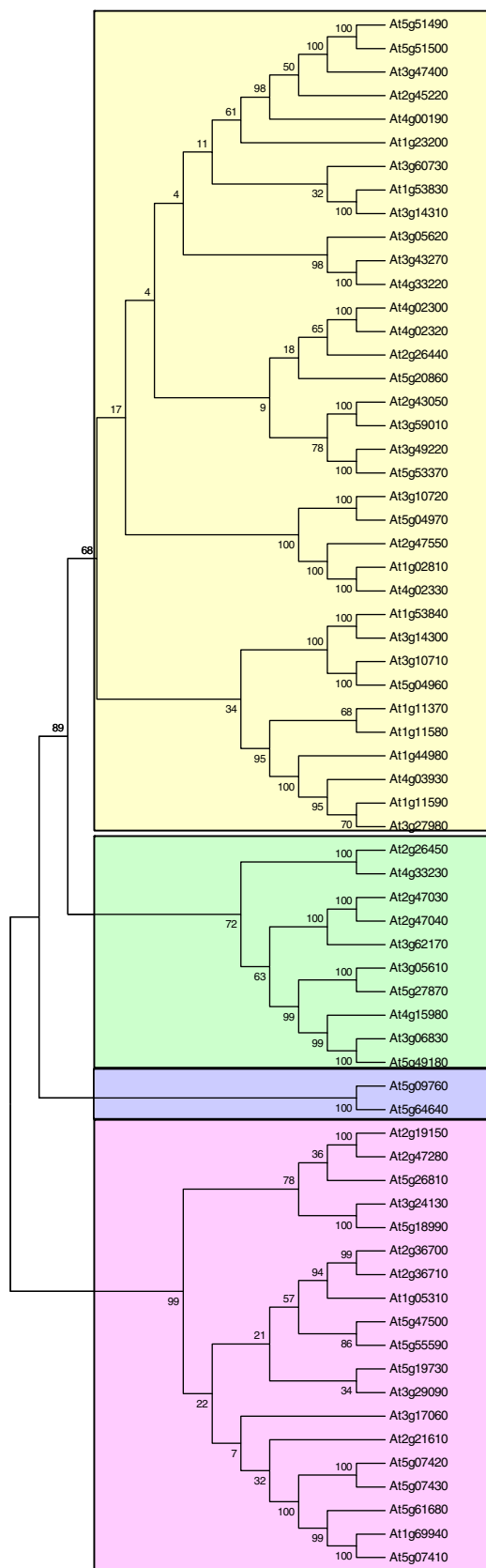


Fig. 3 Phylogenetic analysis of the PME protein sequences. Amino-acid sequences were aligned using ClustalX and the phylogenetic tree was drawn using the molecular evolutionary genetics analysis (MEGA) package with the neighbour-joining method. Bootstrap values are indicated and are calculated for 1,000 replicates. The analysis indicates that four subgroups (1, 2, 3, 4) can be separated on the basis of the robustness of the node

PME expression in developing seeds

As described previously, among the phylogenetic group 2, all but one *PME*, *At5g49180*, had a preferential expression in floral bud. Real-time RT-PCR expression of *At5g49180* transcripts was shown to be high in young siliques, between 1–2 and 7–8 DAF, showing a peak at 3–4 DAF (Fig. 2 and Supplementary material 3). Using a GUS fusion, we were able to confirm the pattern of *At5g49180* promoter activity (Fig. 4d), which was particularly high in young seed (1–2 to 3–4 DAF, Fig. 4e). Expression was notably present in the micropyle area of the ovule just after fertilization (Fig. 4f). From this data, it is difficult to determine whether the expression is restricted to the embryo, but our data suggest that *At5g49180* could be involved in the early events of seed

Group 1

Group 2

Group 3

Group 4

Table 1 Relation between phylogenetic groups and expression profiles. Expression values of *PME* were analysed with regard to the phylogenetic tree for group 2 and part of group 4, showing a relationship between expression and sequences similarities. Phylogenetic groups were that of Fig. 3. PME expression data, shown as the ratio [PME/ACTIN 2-7-8]×1,000, are extracted from Supplementary material 3

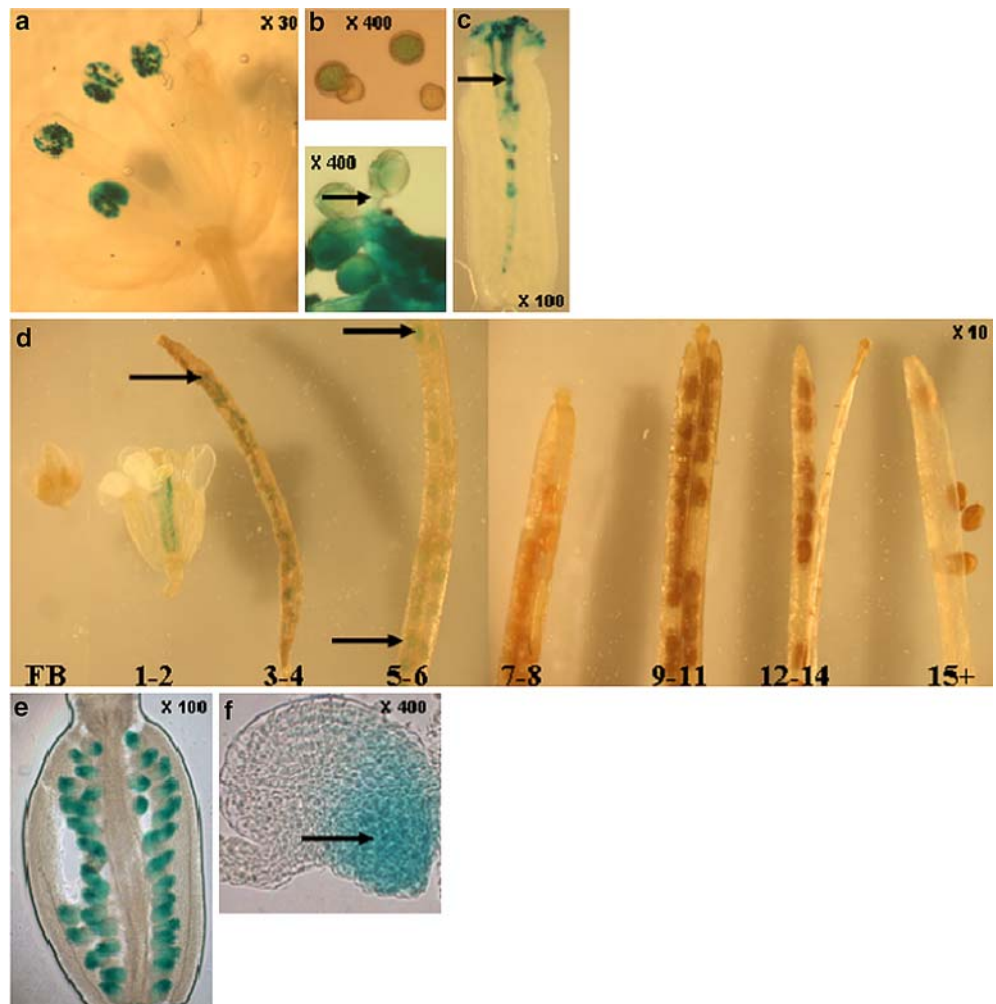
Group2	V	FB	Developmental stages (DAF)						
			1–2	3–4	5–6	7–8	9–11	12–14	15+
At2g26450	×	3.50	0.56	0.64	0.24	×	0.00	×	×
At4g33230	0.01	1.78	0.12	0.04	0.03	×	0.01	×	×
At2g47030	0.04	8.58	2.06	1.02	0.11	0.24	0.64	0.47	0.09
At2g47040	0.71	273.63	26.30	8.79	8.35	1.46	12.05	11.58	1.62
At3g62170	0.44	428.83	11.35	4.84	2.87	0.58	1.09	0.89	0.86
At3g05610	×	0.18	0.02	0.01	×	×	×	×	×
At5g27870	×	0.04	×	×	×	×	×	×	×
At4g15980	×	16.40	0.08	0.02	×	×	×	×	×
At3g06830	0.60	10.69	0.53	0.44	1.00	0.74	0.95	1.03	0.62
At5g49180	×	×	0.82	4.78	4.08	0.30	×	×	×

Group4	V	FB	Developmental stages (DAF)						
			1–2	3–4	5–6	7–8	9–11	12–14	15+
At3g17060	0.11	6.58	0.63	1.13	0.28	0.07	×	×	×
At2g21610	×	×	×	×	×	×	×	×	×
At5g07420	0.16	129.81	3.02	1.09	0.57	0.10	0.16	0.25	0.26
At5g07430	0.28	101.81	7.81	3.54	1.10	0.31	0.50	0.29	0.27
At5g61680	×	0.01	0.01	0.01	×	×	×	×	×
At1g69940	×	83.79	×	×	×	×	×	×	×
At5g07410	0.16	16.84	×	×	×	×	×	×	×

Code is as follows: × no expression, *normal values* 0 to 1, *italics* 1 to 10, *bold* 10 to 100, *bold italic* 100 to 1,000

V Vegetative control, FB Floral bud, 1–2 to 15+ 1–2 to 15+ days after flowering

Fig. 4 Promoter activity of *At1g69940* and *At5g49180* monitored by GUS activity. **a** *PrAt1g69940:GUS* expression showing high promoter activity in anthers and especially in pollen grains (**b**). **c** *PrAt1g69940:GUS* expression was sustained in pollen tubes growing within the style (*arrow*). **d** *PrAt5g49180:GUS* expression during silique development showing promoter activity up to stage 5–6 DAF (*arrow*). **e**, **f** *PrAt5g49180:GUS* at stage 1–2 DAF showing that promoter activity (*arrow*) is restricted to the central area of the newly fertilized ovule



development in *Arabidopsis*. Similar RT-PCR quantification and promoter–GUS staining were found for *At1g11590* (data not shown), belonging to phylogenetic group 1 and expression group E.

Discussion

The expression of the entire *Arabidopsis* PME gene family was measured during silique development. A phylogenetic analysis of the gene family showed that *Arabidopsis* PMEs could be clustered into four distinct groups, and that one, group 4, was restricted to PMEs without PRO part. This sustained the common hypothesis that plant PMEs with a PRO region are likely to have evolved later (Micheli 2001). When compared to the tree of Markovic and Janecek (2004), based on 58 sequences, our analysis showed a reduced number of groups (from 7 to 4), mainly due to the fact that a bootstrap level of 50% was used. Despite these differences, group 3 was similar to group PlantX1, and very distinct from all the other groups in both studies, which could imply a specific role for the products of those two genes during plant

development. This tree served as a basis for further comparisons between *PMEs* expression patterns and *PME* protein sequences.

PMEs expression showed a high diversity of profiles during silique development. Such diversity of expression patterns was obtained for 88 MADS-box transcription factors (de Folter et al. 2004) as well as for *PMEs* during fruit development in a wide range of species. In strawberry, *FaPE1* was uniquely expressed in the ripening fruit, whereas *FaPE3* was ubiquitous (Castillejo et al. 2004). In green bean, *PE2* was very abundant during early pod development (Ebbelaar et al. 1996). In tomato, *PE2* was a fruit-specific isoform highly expressed 25 days after anthesis (Eriksson et al. 2004) and in grape berry, specific *PMEs* were expressed from veraison stage onwards (Barnavon et al. 2001). Although the processes involved in fleshy and dry fruit development are different, it is likely that in both cases, *PMEs* could play a major role in modifying pectins leading to fruit maturation. Moreover, for a similar expression pattern, our study pointed out the high differences of expression levels between isoforms (expression group D or E for instance). Such differences were notably shown during silique development in *Arabidopsis* for the MADS-box

family using microarray analysis, varying, for instance, between 0.46 and 11.04 for *AGL89* and *SEP2*, respectively (de Folter et al. 2004). A discrepancy between expression ratio was shown between real-time RT-PCR and microarray approaches during the study of the expression of all transcription factors families: for instance for *COL7*, the shoot to root (S/R) expression ratio was 8,678 using RT-PCR and 101 using microarray (Czechowski et al. 2004). In our study, differences were obtained when comparing our RT-PCR results with some of the expression data freely available on the TAIR website (<http://www.arabidopsis.org>). For instance, *At5g19730* had a 3–4 DAF to vegetative expression ratio of 143, whereas the globular to vegetative expression ratio was only 1.3 from microarray data. Similar discrepancies were observed, for example, for *At3g17060* and *At1g11590*. The use of real-time RT-PCR significantly increases the sensitivity, accuracy and precision of the measurement, especially when dealing with a multigene family (Czechowski et al. 2004).

The low total *PME* expression at stage 7–8 was concomitant with the arrest in maximum length growth of both the silique and the embryo (Baud et al. 2002). Stage 7–8 DAF could be the *PME* expression shift point between isoforms involved in either length growth or silique maturation. Although a high transcript level does not necessarily translate into a high level of protein and enzyme activity, a role for *PME* in silique growth and differentiation could be suspected. A study on green bean did show that *PME* activity dropped at stage III, where the maximum length of the pod was reached, before increasing during pod maturation (Stolle-Smits et al. 1999). The increase in *PME* transcript quantities (notably *At3g60730*, *At5g51490*) in the late developmental stages of the silique (9–11 onwards) could increase *PME* activity. This would lead to a modification of the pectic network through the action of both *PME* and polygalacturonases (PG), enzymes involved in fruit dehiscence (Roberts et al. 2000). The analysis of homogalacturonans methylesterification patterns (blockwise or non-blockwise), and of *PME* activity regulation (ionic interaction, pH...) during silique development will help dissecting the role of the pectin network remodelling during fruit development.

Among the ubiquitously expressed *PMEs* identified in our study, microarray data analysis showed that *At4g02330* and *At3g49220* expressions in seedlings were positively regulated by brassinosteroid and ethylene, respectively (Goda et al. 2002; De Paepe et al. 2004). This suggests a role for plant growth regulators in the signalling cascade leading to *PME* transcript regulation. The potential diversity of the roles of putative *PMEs* in modulating cell wall structure in plant development could be further illustrated by *At2g45220*, which was notably expressed in vegetative tissues. Microarray analysis showed that *At2g45220* expression was regulated by anoxia (Loreti et al. 2005), by auxin (Goda et al. 2004), by cold (Kreps et al. 2002) as well as during the weight-induced secondary growth in *Arabidopsis* (Ko et al.

2004). Numerous other putative *PMEs*, such as *At1g02810*, *At2g43270* and *At3g10720*, which were all expressed in our vegetative control, were upregulated during cell differentiation leading to wood formation in *Arabidopsis* (Ko et al. 2004). In a recent study, *At2g43270* and *At3g14310* were both shown to be highly expressed in phloem when compared to xylem and non-vascular secondary tissues of root-hypocotyl (Zhao et al. 2005), suggesting that those isoforms could play a role in conducting element differentiation.

As shown for *At2g45220*, several *PMEs* are also likely to be involved in stress response. For instance, *At1g11580* expression, belonging to our expression group A, was notably shown to be induced ($\times 5.3$) by the herbicide isobaxen in suspension cultured cells (Manfield et al. 2004) as well as in distal leaf tissues after 72 h inoculation with the incompatible fungal pathogen *Alternaria brassicicola* (Schenk et al. 2003). Such *PME* transcript regulation was shown in *Medicago* following inoculation of roots with *S. meliloti* (Rodriguez-Llorente et al. 2004), showing that the production of *PMEs* following biotic interaction might be widespread.

In our study, a correlation was shown between expression profiles and the phylogenetic tree, notably for group 2 and a subset of group 4. Such expression-to-sequence relationship was previously shown for some members of the MIKC subset of the MADS-box TF family (de Folter et al. 2004). Although the majority of the *PMEs* of these two groups (2 and 4) were previously shown to be highly expressed in pollen (Pina et al. 2005), our study brings a new insight into the expression profiles of those genes. For instance, *At2g47040*, which encodes VANGUARD1 (Jiang et al. 2005) is indeed very highly expressed in flower bud (273), but also throughout the entire silique developmental process, although with a smaller intensity (up to 26). Microarray data analysis showed a similar expression pattern, which suggests that the role of *At2g47040* in pollen tube growth in the style and transmitting tract, as shown in knock-out mutant, might therefore not be the only one for this protein, since the mutant had also smaller silique with few seeds (Jiang et al. 2005). Our results suggest that this latter phenotype could either be related to the low ovule fertilization (linked to the expression of *VGD1* in pollen) or to an independent phenotype due to the lack of *VGD1* expression in the silique. The relative *At2g47040* to *At2g47030* expression ratio was 34 in our experiment, which was in the same magnitude as the one shown by Jiang et al. (2005), but could not be compared with microarray data as *At2g47030* is one of the 6 *PMEs* which is not available on the Affimetryx ATH1 genome array. A recent study on pollen transcriptome revealed that among the *PMEs* found to be expressed in floral bud in our study (expression group D), 11 out of 19 had a relative expression level above 1,000 in microarray experiments (Pina et al. 2005). Our study further revealed that *At1g69940*, a *PME* without PRO part, which is not present on the microarray data presented by Pina et al. (2005) showed an expression pattern restricted to floral bud, and more

precisely in pollen grains using promoter–GUS fusions. In that respect, the localisation of expression of *At1g69940* was similar to that shown for *At2g47030*, *At2g47040* and *At3g62170* (Jiang et al. 2005). It is very likely that a high redundancy of PME expression exists in pollen. Numerous studies have shown that PME or PME-like genes were expressed in pollen, notably in flax (Lacoux et al. 2003), in *Zea* (Wakeley et al. 1998) and in *Nicotiana* (Bosch et al. 2005). However, it is not until recently that the role of PME as a regulator of pollen tube growth, suspected by Parre and Geitmann (2005) in *Solanum chacoense*, was demonstrated in tobacco (Bosch et al. 2005). The role of *At1g69940*, among the other pollen-expressed PMEs, in the control of homogalacturonan esterification pattern, therefore, needs to be addressed using a reverse genetic approach. In that context, it would also be of interest to study the regulation of these numerous pollen-expressed PMEs by any of the two PME inhibitors, *At-PMEII* and *At-PMEL*, present in pollen (Wolf et al. 2003).

Within phylogenetic group 2, only one PME differed from the general floral bud expression pattern. *At5g49180* expression clustered with that of *At1g11590*, *At4g02300* and *At4g03930*, showing a specificity of expression during early stages of silique development, up to stages 7–8 DAF, and specifically in seed for *At5g49180* and *At1g11590* (data not shown). While microarray data analysis confirmed high expression for *At5g49180* at globular/torpedo stages, no such results were available for the three other members of our expression group E (<http://www.arabidopsis.org>). *At1g11590* and *At4g03930* promoters were 87% identical, containing GARE motifs necessary for gibberelin-upregulated expression in seeds (Suto and Yamauchi 2003). *At4g02300* and *At5g49180* promoters contained embryo-specific expression motifs (Kim et al. 1997). Thus, cluster group E is likely to contain young seed-specific PMEs. Activities of PMEs increased in yellow cedar seeds at the onset of dormancy breakage and germination (Ren and Kermodé 2000) as well as in flax during early seedling development (Alexandre et al. 1997). Moreover, in soybean seeds, pectin esterase activity had a biphasic phase with a maximum in axes at 24 DAF, a minimum at 56 DAF and an increase during desiccation phase (Koch et al. 1999). A biphasic expression was shown for PME transcripts during silique development in our study, and genes, such as *At5g49180*, *At1g11590*, *At4g02300*, could contribute to a first peak of PME activity which might be involved in the early event of embryo/seed development.

Conclusion

The expression kinetics of an entire cell wall gene family during silique development in *Arabidopsis* was determined. The expression of most PMEs was detected in flowers or siliques, and the gene family could be split into five groups according to their expression patterns.

Correlations were made between expression profiles and phylogenetic analysis, showing that closely related sequences could have similar expression kinetics. Real-time RT-PCR studies, together with promoter–GUS fusions will allow the functional genomic approach to be targeted on candidates important during specific silique developmental stages. This will help the precise function of the various PMEs to be determined.

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