Two *Arabidopsis thaliana* genes encode functional pectin methylesterase inhibitors[1]

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Abstract **We have identified, expressed and characterized two genes from *Arabidopsis thaliana* (*AtPMEI-1* and *AtPMEI-2*) encoding functional inhibitors of pectin methylesterases. AtPMEI-1 and AtPMEI-2 are cell wall proteins sharing many features with the only pectin methylesterase inhibitor (PMEI) characterized so far from kiwi fruit. Both Arabidopsis proteins interact with and inhibit plant-derived pectin methylesterases (PMEs) but not microbial enzymes. The occurrence of functional PMEIs in Arabidopsis indicates that a mechanism of controlling pectin esterification by inhibition of endogenous PMEs is present in different plant species.**

Key words: Plant cell wall; Pectin; Pectin methylesterase; Pectin methylesterase inhibitor; *Arabidopsis thaliana*

1. Introduction

Pectin, one of the main components of the plant cell wall, is secreted into the wall in a highly methylesterified form and subsequently de-esterified in muro by pectin methylesterase (PME, E.C. 3.1.1.11) [1]. The de-esterification is the prerequisite for pectin degradation by either plant or microbial enzymes. PMEs are involved in important developmental processes such as fruit ripening, microsporogenesis, tube pollen growth, seed germination and hypocotlyl elongation [1]. PMEs are also produced by phytopathogenic microorganisms during plant infection and by symbiotic microorganisms during their interactions with plants [2]. The complex role of PME in the dynamics of the plant cell wall is indicated by the presence of 67 PME-related genes in the Arabidopsis genome [1].

A possible control of PME activity by inhibitors was hypothesized when a protein inhibitor (PMEI) was discovered in kiwi (*Actinidia chinensis*) fruit [3–5]. To date, kiwi PMEI homologs have not yet been discovered in other plant species. We have identified two Arabidopsis genes encoding proteins closely related to kiwi PMEI. Both proteins, expressed in *Pichia pastoris*, have been shown to be functional inhibitors of plant-derived PMEs. Our results indicate that these inhibitors are present in different plant species and inhibition of PME may be an important way of controlling pectin esterification.

2. Materials and methods

2.1. Materials

Arabidopsis thaliana seeds, accession Columbia (Col-0), were obtained from the Arabidopsis Information Service (Germany). Plants were grown at 22°C with a 12-h day/night cycle. Arabidopsis cell cultures were grown at 25°C on a rotary shaker at 80 rpm under a 16-h day/night cycle. Cells were subcultured every 3 weeks in Murashige and Shoog liquid medium supplemented with 30 g/l sucrose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid and 0.25 mg/l 6-benzylaminopurine. PME from *Erwinia chrysanthemi* and sugar beet pectin (93% methylated) was a gift from Danisco Innovation, Denmark; citrus pectin (63–66% methylated) was obtained from Fluka; PME from *Aspergillus niger* was obtained from Sigma and PME from *Aspergillus aculeatus* was a gift from Novozyme A/S (Denmark).

2.2. Cloning and expression of AtPMEI-1 and AtPMEI-2

Genomic DNA was isolated from Arabidopsis seedlings using the NucleoSpin Plant Kit from Macherey-Nagel, Germany. *AtPMEI-1* and *At1PMEI-2* were amplified by polymerase chain reaction (PCR) from genomic DNA (50 ng) with PWO-DNA polymerase (Roche) using the following primer pairs: *AtPMEI-1/F* (5′-ATCGAGAATTCATCACAAGTTCAGAAATGAG-3′); *AtPMEI-1/R* (5′-ATCGATCTAGATTAATTACGTGGTAACATGT-3′); *AtPMEI-2/F* (5′-ATCGAGAATTCCAAGTGGCAGACATAAAAG-3′); *AtPMEI-2/R* (5′-ATCGATCTAGATCACATCATGTTTGAGATG-3′).

The amplification products were isolated and cloned between the *Eco*RI and *Xba*I sites (underlined in the above primer sequences) into the pPICZαA vector and used to transform *P. pastoris* strain X-33 according to the Pichia EasyComp™ transformation kit (Invitrogen). Transformed *P. pastoris* cells, grown to saturation in BMGY medium (Invitrogen) at 28°C with shaking at 250 rpm, were harvested by centrifugation, resuspended in a modified BMMY medium (0.4% (w/v) yeast extract, 0.6% (w/v) tryptone, 100 mM potassium phosphate pH 6.0, 1.34% (w/v) yeast nitrogen base (YNB), 0.4 g/ml biotin, 1% (v/v) methanol) and grown for 60 h. Methanol, to a final concentration of 0.5% (v/v), was added every 24 h. After centrifugation at $10\,000\times g$ for 15 min the supernatant of the culture was collected and stored at −80°C.

2.3. Purification and characterization of AtPMEI-1 and AtPMEI-2

PME was purified to homogeneity from tomato and assayed as

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[1] The industrial utilization of Arabidopsis and kiwi PMEIs is patent pending It, no. RM2003A000346.
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Abbreviations: PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; SPR, surface plasmon resonance

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reported [6]. N-terminal sequencing provided identification of the PME isoform as Lyces_PME1 (SwissProt accession number P14280). PMEI from kiwi fruit was prepared and assayed as reported [4].

The supernatants from cultures of *P. pastoris* expressing AtPMEI-1 and AtPMEI-2 were treated with ammonium sulfate (80% saturation) and centrifuged at 10 000×*g*. The precipitate containing AtPMEI-2 was dissolved in 10 mM Na acetate, pH 5.0, extensively dialyzed and loaded onto a MonoS column (HR 10/10, Pharmacia). The precipitate containing AtPMEI-1 was dissolved in 10 mM Tris–Cl, pH 8.5 and loaded onto a MonoQ column (HR 10/10, Pharmacia). Both columns were eluted by a linear gradient of NaCl from 0 to 0.5 M at a flow rate of 1.5 ml/min on a Pharmacia fast protein liquid chromatography (FPLC) system. The active fractions were pooled, concentrated by ultrafiltration on Centricon 3 filters (Amicon) and separately loaded onto a Sephadex G75 column (1.5×90 cm), equilibrated in 10 mM Tris–Cl, pH 7.5, 250 mM NaCl at a flow rate of 5 ml/h. The purity of the eluted proteins was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), by high-performance liquid chromatography (HPLC) onto a reverse phase column (Vydac C4, 4.6×250 mm, 5 μ) and amino acid sequencing analysis on a Procise Sequencer model 492 from Applied Biosystems.

Protein concentration was determined by a modified Lowry method from Bio-Rad or by measuring the absorbance at 280 nm, using the extinction coefficient at 1% and 1 cm of 1.72 (AtPMEI-1) and 2.61 (AtPMEI-2).

Circular dichroism measurements were performed as reported [5].

Homology search was performed by using BLAST and sequences were aligned using DNAman Lynnon Biosoft software. Molecular weight, isoelectric point and extinction coefficient were calculated by ProtParam Tool; *N*-glycosylation sites were predicted by ScanProsite (http://www.expasy.org). The signal peptide cleavage site was predicted by SignalP program (http://www.cbs.dfu.dk/service/SignalP). Secondary structure predictions were obtained by SOPMA [8]. The hydropathy was predicted by DNAman program [9].

Inhibitory activity of PMEI was assayed by an agar diffusion assay as described [10], using a 93% methylated pectin at pH 6.3. PME aliquots producing a 1-cm diameter stained zone (about 15 ng of purified tomato PME, in a standard assay) and different aliquots of PMEI were used. The pH dependence of PMEI was assayed by the titrimetric method. The minimal amount of each PMEI necessary to completely inhibit a fixed amount of tomato PME at pH 6.5 was defined, and the same amount of inhibitor was assayed at pH 7.5 and 8.5. For temperature stability analysis, the same amount of each PMEI was incubated for 10 min at the indicated temperatures and then assayed at room temperature. For surface plasmon resonance (SPR) analysis homogeneous tomato PME was immobilized to sensor chip CM5 (Biosense) in a BIAcoreX system. The interaction with purified AtPMEIs was analyzed as previously described [7]. The invertase inhibitory activity was determined according to Scognamiglio et al. [11].

2.4. RNA gel blot analysis

RNA was extracted from Arabidopsis vegetative tissues and flowers as reported [12]. Hybridization probes were obtained by random oligonucleotide priming of a PCR-amplified coding region of each *AtPMEI* gene.

3. Results and discussion

NCBI database search revealed two Arabidopsis sequences (At1g48020 and At3g17220) closely related to the kiwi PMEI protein sequence (AcPMEI; accession number P83326) and predicted to belong to the invertase/PMEI family. Both At1g48020 and At3g17220 sequences will be shown to encode functional PMEIs, hereafter named AtPMEI-1 and AtPMEI-2 respectively.

AtPMEI-1 and AtPMEI-2 share about 38% identity at the amino acid level with AcPMEI, and 64 and 45% identity at the nucleotide and amino acid level, respectively, with each other. Removal of the predicted N-terminal signal peptide generates mature proteins of 151 amino acids (molecular mass of 16 266 Da, predicted p*I* = 7.7) and 148 amino acids (molecular mass of 15 615 Da, predicted p*I* = 9.0). AtPMEI-1 has one potential *N*-glycosylation site at position 129 and AtPMEI-2 has two potential *N*-glycosylation sites located at positions 14 and 38 (Fig. 1). Like AcPMEI, both Arabidopsis proteins show a conserved C-terminal hydrophobic region (CxIxLVISN) and five conserved Cys residues, the first four of which have been shown to be engaged in disulfide bridges in AcPMEI [5]. Secondary structure predictions indicate a high content of α-helix structure for both Arabidopsis proteins (61.6 and 63.5% for AtPMEI-1 and AtPMEI-2, respectively). The hydropathy profiles of the Arabidopsis inhibitors were similar to that of AcPMEI along the entire sequence and almost overlapping with the exception of a less hydrophobic domain in the region between residues 70 and 80 of AcPMEI (not shown).

Search for Arabidopsis sequences related to AtPMEI-1 and AtPMEI-2 indicated a number of proteins with an identity level higher than 20% and showing conserved Cys residues like in AcPMEI [5,11]. A dendrogram generated with these sequences and including AcPMEI and tobacco invertase inhibitors (Nt-VIF and Nt-CIF) [13,14] identifies three independent protein groups. The first group includes AcPMEI, AtPMEI-1/At1g48020 and AtPMEI-2/At3g17220. The second group includes Nt-VIF, Nt-CIF and the Arabidopsis At1g47960 protein, a probable invertase inhibitor. The last group is formed by five members with unidentified function, all located on chromosome 5 (Fig. 2).

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AcPMEI      ---------------------------ENHLISEICPKTRNPSLCLQALESDPRSASKDLK
AtPMEI-2    MAAYLTNRVLMSSLMFFVMTGSLNAQVADIK-AICGKAKNQSFCTSYMKSNPKTSGADLQ
AtPMEI-1    MAANLRNNAFLSSLMFLLLIGSSYAITSSEMSTICDKTLNPSFCLKFL--NTKFASPNLQ
                                            ** *. * * *   .    . .   *.

AcPMEI      GLGQFSIDIAQASAKQTSKIIASLTNQATDPKLKGRYETCSENYADAIDSLGQAKQFLTS
AtPMEI-2    TLANITFGSAQTSASEGFRKIQSLVKTATNPTMKKAYTSCVQHYKSAISSLNDAKQSLAS
AtPMEI-1    ALAKTTLDSTQARATQTLKKLQSIIDGGVDPRSKLAYRSCVDEYESAIGNLEEAFEHLAS
             *   .   .*. * .  . . *.      *  *  * .*   *  **  *  * . *.*

AcPMEI      GDYNSLNIYASAAFDGAGTCEDSFEGPPNIPTQLHQADLKLEDLCDIVLVISNLLPGS
AtPMEI-2    GDGKGLNIKVSAAMEGPSTCEQDMADFK-VDPSAVKNSGDFQNICGIVLVISNMM
AtPMEI-1    GDGMGMNMKVSAALDGADTCLDDVKRLRSVDSSVVNNSKTIKNLCGIALVISNMLPRN
            **   .*.  *** .*  **         .              .* * *****.
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Fig. 1. Amino acid sequence alignment of AtPMEI-1 and AtPMEI-2 with AcPMEI. Asterisks and dots indicate 100 and 66% amino acid identity, respectively. The five conserved Cys residues and the putative *N*-glycosylation sites are gray shadowed. Signal sequence of Arabidopsis proteins is underlined. The conserved C-terminal hydrophobic region is in bold.

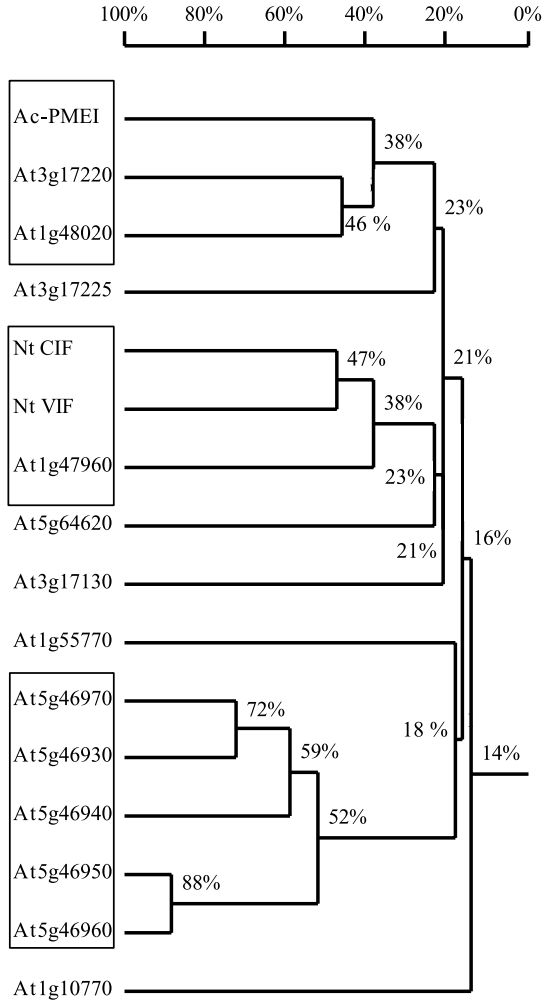

Fig. 2. Homology tree of several Arabidopsis protein sequences and the functionally characterized kiwi PMEI (AcPMEI) and apoplastic (Nt CIF) and vacuolar (Nt VIF) tobacco invertase inhibitors. Multiple sequence alignment was performed using DNAman software package (Lynnon Biosoft). Numbers at branch points represent average identities.

The intronless regions of *AtPMEI-1* and *AtPMEI-2* encoding the predicted mature proteins were amplified by PCR using genomic DNA from Arabidopsis seedlings as a template. The amplified fragments were cloned and expressed in *P. pastoris*, which produced about 20 mg/l of both proteins in the culture filtrate. The proteins encoded by *AtPMEI-1* and *AtPMEI-2* were purified as reported in Section 2. In order to confirm the protein identities N-terminal sequences were carried out. AtPMEI-1 had the N-terminal sequence EAEFITSSEMSTI-D, where position 14 yielded a blank cycle due to the presence of a Cys residue. AtPMEI-1 showed a molecular mass of 18 000 Da by SDS–PAGE (Fig. 3) and a single peak with a calculated molecular mass of 22 000 Da on gel filtration in native conditions. AtPMEI-2 yielded the sequence EAEFQVADIKAI-GKAK-Q, where the blank cycle at positions 13 is due to a Cys residue while the blank cycle observed at position 18 corresponds to the first *N*-glycosylation site of the protein and indicates its actual glycosylation. The purified protein showed on SDS–PAGE a single band with an apparent molecular mass of 25 000 Da (Fig. 3) and a single peak with a calculated molecular mass of 29 500 Da on gel filtration in native conditions. The high molecular masses observed by SDS–PAGE are probably due to the differential glycosylation of the two proteins. A complete deglycosylation of the inhibitors could not be obtained by treatment with endoglycosidase H, although the mobility of the partially deglycosylated proteins considerably increased in SDS–PAGE. The comparison of SDS–PAGE performed in reducing and not reducing conditions showed a shift of both non-reduced proteins towards lower molecular mass values indicating that the cysteine residues are engaged in disulfide bridges, as in the case of PMEI from kiwi (not shown).

The activities of AtPMEI-1 and AtPMEI-2 were assayed by gel diffusion assay against various PMEs. AtPMEI-1 was active against the main PME isoform purified from tomato fruit and against the enzymes from kiwi and apricot fruits, carrot roots, tobacco leaves, Arabidopsis leaves and flowers whereas it was inactive against PMEs of *E. chrysanthemi*, *A. niger* and *A. aculeatus* (Fig. 4). AtPMEI-2 showed an identical behavior (data not shown). Both inhibitors were inactive against invertase from tomato fruit in agreement with the results of other authors [15]. The specific inhibitory activity of AtPMEI-1 and AtPMEI-2, determined at pH 7.5 against tomato PME, was 100 and 500 IU/mg as defined in [3], respectively. Endoglycosidase H treatment reduced the molecular mass of the inhibitors, without affecting their activity (not shown). AtPMEI-2 was active against tomato PME in the pH range 6.5–8.5, AtPMEI-1 was less active at pH 8.5 while AcPMEI was completely inactive at pH 8.5 (Table 1). SPR analysis of tomato PME–AtPMEIs interactions at a pH in the range 5.5–8.5 showed the absence of dissociation for AtPMEI-2, and a k_{off} of 1.1×10^{-3} and a K_D of 5.5 nM for AtPMEI-1 at pH 8.5 (Fig. 5), while no dissociation occurred at lower pH values. Both Arabidopsis inhibitors showed a better thermal stability than the kiwi inhibitor (Table 2).

The circular dichroism spectra of the two proteins, recorded in the range 240–195 nm, showed a minimum at 222 nm and a second minimum at 208 nm, typical of the α-helix structure;

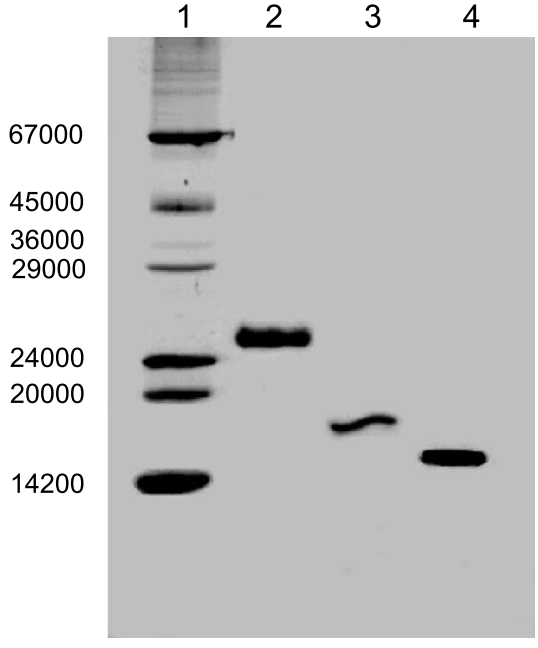

Fig. 3. SDS–PAGE analysis of purified PMEIs. (1) Molecular weight standard, (2) AtPMEI-2, (3) AtPMEI-1, (4) kiwi fruit PMEI.

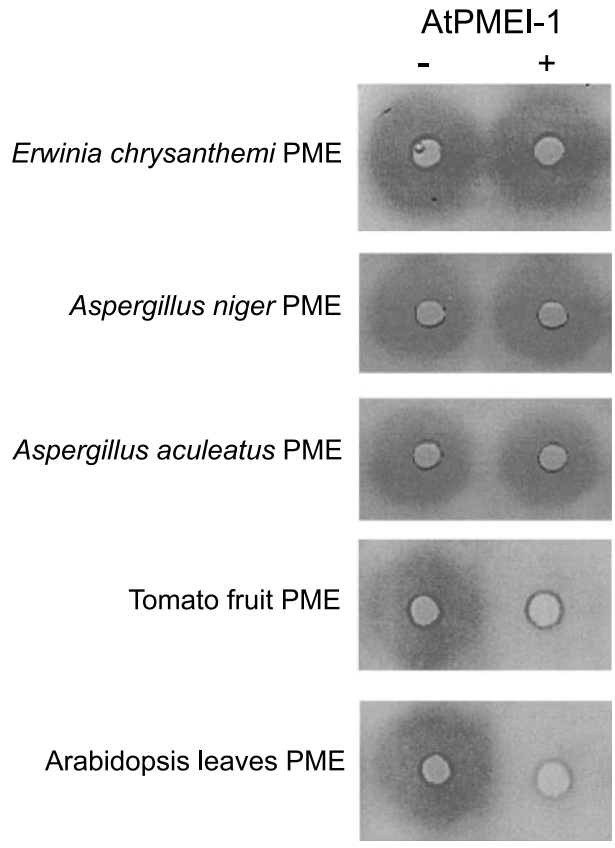

Fig. 4. Gel diffusion assay showing the inhibitory activity of AtPMEI-1 against various PMEs.

the spectra were almost super-imposable with that of the kiwi PMEI. The estimated percentage of α-helix was 65% in agreement with the high percentage of α-helix predicted on the basis of amino acid sequences of AtPMEI-1, AtPMEI-2 and AcPMEI. The stoichiometry of the AtPMEI/PME interaction was determined by detecting the peak shift in size exclusion chromatography experiments on Superdex 75 at pH 6.5. If a 1:1 mixture was applied to the column a single peak was visible, indicating the presence of a highly stable complex, which did not dissociate during the gel filtration chromatography. When the AtPMEI:PME ratio was higher than 1, the excess of AtPMEI was visible in addition to the peak corresponding to the bimolecular complex. Thus it was concluded that AtPMEIs, like AcPMEI, form 1:1 complexes with plant PMEs [4,16].

The steady-state messenger RNA levels of the AtPMEI genes were examined in various tissues by Northern blot analysis. Both *AtPMEI-1* and *AtPMEI-2* genes were highly expressed in Arabidopsis flowers; in addition, *AtPMEI-1* mRNA was detected in leaves of mature plants, albeit at a lower level. Transcripts of both genes were not detected in cultured cells or the other vegetative tissues analyzed (Fig. 6).

Table 1
Inhibitory activity towards tomato PME as a function of pH

	Inhibitory activity[a] (%)			
	pH 5.5	pH 6.5	pH 7.5	pH 8.5
AtPMEI-1	100	100	100	60
AtPMEI-2	100	100	100	100
AcPMEI	100	100	75	0

[a]Activity was detected by the titrimetric assay. The inhibition observed at pH 6.5 was taken as 100%.

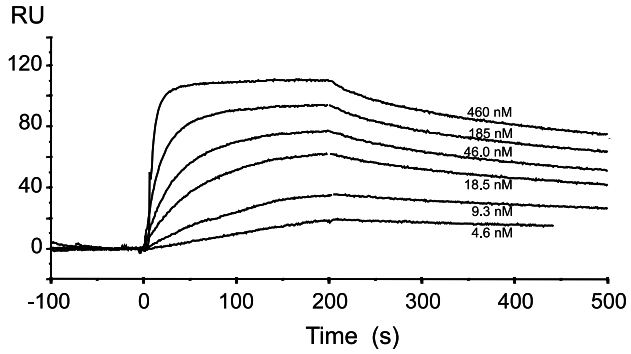

Fig. 5. SPR analysis of tomato PME and AtPMEI-1 interaction at pH 8.5. After each measuring cycle, several injections of 0.1 M CAPS buffer pH 10 were necessary to regenerate the surface.

4. Conclusions

Plants synthesize protein inhibitors against pectic enzymes. In addition to the well-characterized and ubiquitous polygalacturonase-inhibiting proteins [17], a PMEI was originally found in kiwi fruit [3]. Attempts to characterize PMEI activity in tissues other than kiwi were either unsuccessful or led to the isolation of an inhibitor of invertase, which shares some structural similarities with PMEI but has a completely different target enzyme [11,14]. In this work we have identified a small family formed by two Arabidopsis *PMEI* genes located on different chromosomes which encode two active inhibitors. AtPMEIs have structural features similar to AcPMEI and possibly have similar surfaces engaged in the interaction with PME. The most remarkable difference is the higher stability of the complex formed by AtPMEIs in a wider pH range and the higher thermal stability which make the Arabidopsis inhibitors better suited for biotechnological purposes. Like AcPMEI, AtPMEIs specifically recognize PMEs of plant origin and not microbial enzymes, suggesting a possible role of these inhibitors in plant growth and development. The abundant occurrence of *AtPMEI-1* and *AtPMEI-2* transcripts in flower tissues suggests a developmental role of these inhibitors during flower formation or during the reproductive process. On the other hand, the involvement of PMEI in plant–pathogen interactions can not be excluded. It is well known that the systemic movement of tobamovirus through plasmodesmata requires host cell PME [18,19]. Moreover, it is well known that the level and pattern of pectin methyl esterification may determine the level of plant susceptibility to fungal and bacterial pathogens [20,21]. Modulation of PME activity may, therefore, be crucial for defence.

Table 2
Relative activity of PMEIs incubated at different temperatures for 10 min

	Inhibitory activity (%)				
	40°C	50°C	60°C	70°C	80°C
AtPMEI-1	100	75	60	27	12
AtPMEI-2	100	100	89	42	15
AcPMEI	100	100	63	0	0

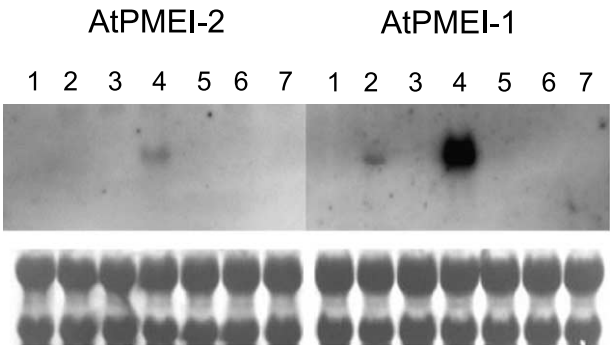

Fig. 6. Analysis of *AtPMEI* mRNA in Arabidopsis tissues. Total RNA gel blot analysis (15 μg RNA per lane) of AtPMEI-1 and AtPMEI-2 expression in: lane 1, seedlings; lane 2, leaves from mature plants; lane 3, roots; lane 4, flowers; lane 5, stem; lane 6, cultured cells in exponential phase; lane 7, cultured cells in stationary phase. The filters in the lower panel, stained with methylene blue to verify equal loading, show the bands corresponding to 26S and 18S rRNA.

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