

Two *Arabidopsis thaliana* genes encode functional pectin methylesterase inhibitors¹

A. Raiola^{a,2}, L. Camardella^{b,2}, A. Giovane^c, B. Mattei^a, G. De Lorenzo^a, F. Cervone^a,
D. Bellincampi^{a,*}

^aDipartimento di Biologia Vegetale, Laboratorio di Genomica Funzionale e Proteomica, Università di Roma 'La Sapienza', Piazzale Aldo Moro 5, 00185 Roma, Italy

^bInstitute of Protein Biochemistry, CNR, Via Marconi 10, I-80125 Napoli, Italy

^cDepartment of Biochemistry and Biophysics, 2nd University of Naples, Via Costantinopoli 16, I-80138 Napoli, Italy

Received 13 November 2003; revised 8 December 2003; accepted 8 December 2003

First published online 23 December 2003

Edited by Ulf-Ingo Flügge

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.

© 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

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 hypocotyl 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 hy-

pothesized 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 *AtPMEI-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'-ATCGA-GAATTCATCACAAGTTCAGAAATGAG-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 *EcoRI* and *XbaI* 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 10000×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

*Corresponding author. Fax: (39)-06-49912446.

E-mail address: daniela.bellincampi@uniroma1.it (D. Bellincampi).

¹ The industrial utilization of *Arabidopsis* and kiwi PMEIs is patent pending It, no. RM2003A000346.

² These authors contributed equally to this work.

Abbreviations: PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; SPR, surface plasmon resonance

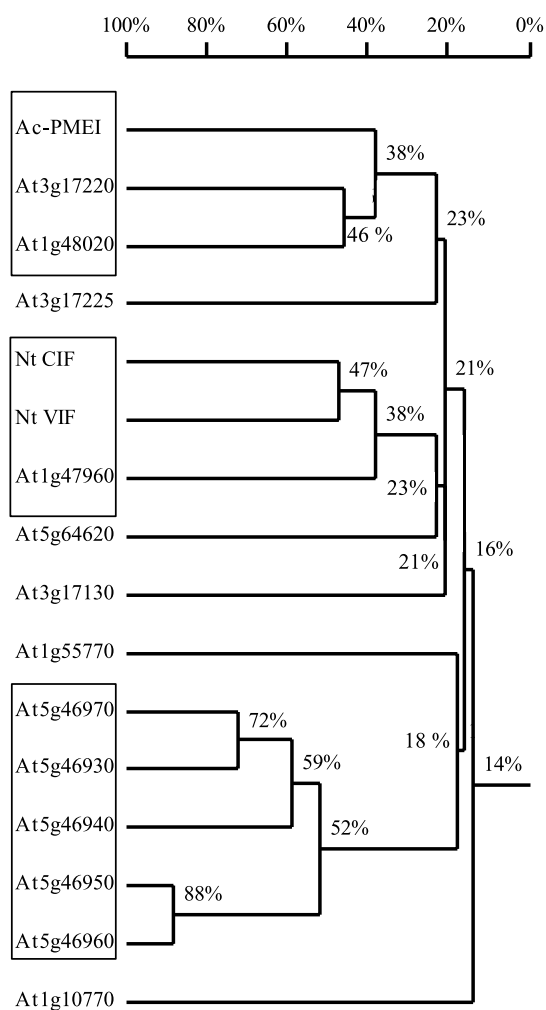


Fig. 2. Homology tree of several Arabidopsis protein sequences and the functionally characterized kiwi PME1 (AcPMEI) and apoplastic (Nt CIF) and vacuolar (Nt VIF) tobacco invertase inhibitors. Multiple sequence alignment was performed using DNAMAN software package (Lynnon Biosoft). Numbers at branch 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 EAEFITS-SEMSTI-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 EAEFQ-VADIKAI-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 PME1 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;

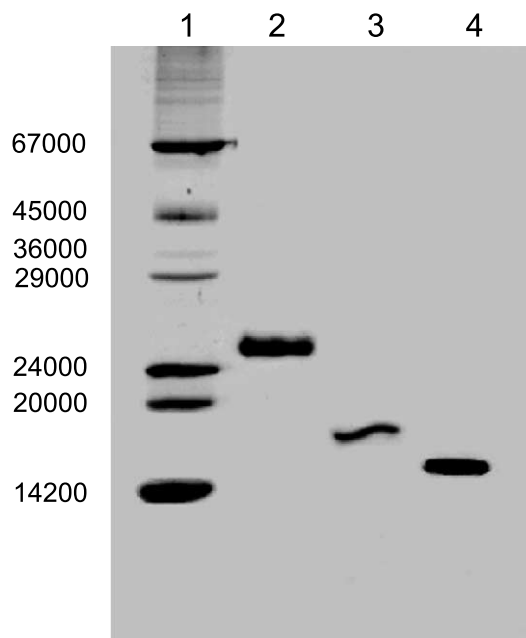


Fig. 3. SDS-PAGE analysis of purified PMEIs. (1) Molecular weight standard, (2) AtPMEI-2, (3) AtPMEI-1, (4) kiwi fruit PME1.

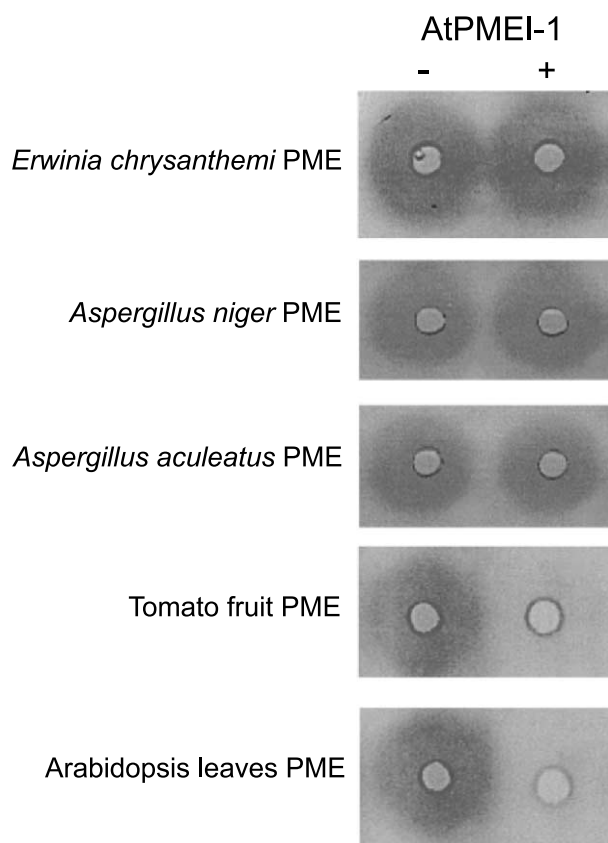


Fig. 4. Gel diffusion assay showing the inhibitory activity of AtPMEI-1 against various PMEs.

the spectra were almost super-imposable with that of the kiwi PME. 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*

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

^aActivity was detected by the titrimetric assay. The inhibition observed at pH 6.5 was taken as 100%.

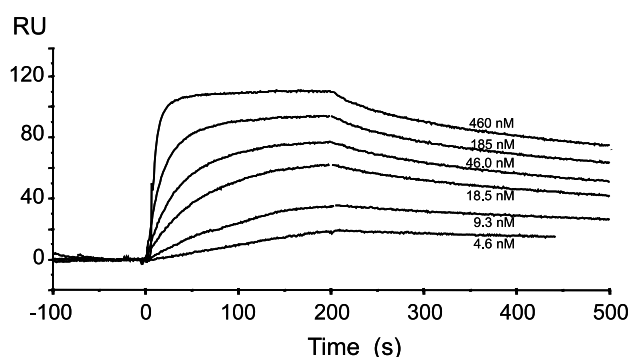


Fig. 5. SPR analysis of tomato PME and AtPMEI-1 interaction at pH 8.5. After each measuring cycle, several injections of 0.1 M CAPS buffer pH 10 were necessary to regenerate the surface.

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).

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

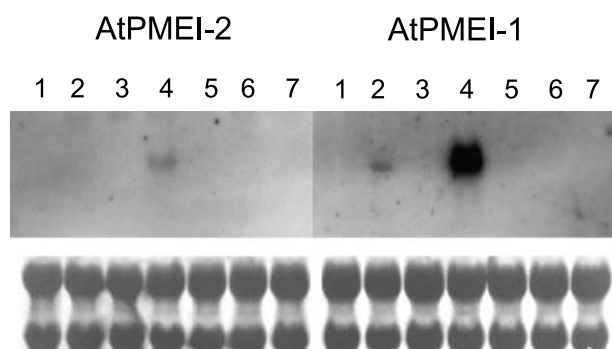


Fig. 6. Analysis of *AtPMEI* mRNA in *Arabidopsis* tissues. Total RNA gel blot analysis (15 µg RNA per lane) of *AtPMEI-1* and *AtPMEI-2* expression in: lane 1, seedlings; lane 2, leaves from mature plants; lane 3, roots; lane 4, flowers; lane 5, stem; lane 6, cultured cells in exponential phase; lane 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.

Acknowledgements: This work was supported by a grant of 'Ministero dell'Università e della Ricerca Scientifica' (COFIN 2002) and by the EU GEMINI project (contract QLK1-2000-00911).

References

- [1] Micheli, F. (2001) *Trends Plant Sci.* 6, 414–419.
- [2] Lievens, S., Goormachtig, S., Herman, S. and Holsters, M. (2002) *Mol. Plant Microbe Interact.* 15, 164–168.
- [3] Balestrieri, C., Castaldo, D., Giovane, A., Quagliuolo, L. and Servillo, L. (1990) *Eur. J. Biochem.* 193, 183–187.
- [4] Giovane, A., Balestrieri, C., Quagliuolo, L., Castaldo, D. and Servillo, L. (1995) *Eur. J. Biochem.* 233, 926–929.
- [5] Camardella, L., Carratore, V., Ciardiello, M.A., Servillo, L., Balestrieri, C. and Giovane, A. (2000) *Eur. J. Biochem.* 267, 4561–4565.
- [6] Giovane, A., Servillo, L., Balestrieri, C., Quagliuolo, L., Laratta, B., Loiudice, R. and Castaldo, D. (1994) *J. Food Biochem.* 17, 339–341.
- [7] Mattei, B., Raiola, A., Caprari, C., Federici, L., Bellincampi, D., De Lorenzo, G., Cervone, F., Giovane, A. and Camardella, L. (2002) in: *Carbohydrate Bioengineering* (Teeri, T.T., Svensson, B., Gilbert, H.J. and Feizi, T., Eds.), pp. 160–167, The Royal Society of Chemistry, UK.
- [8] Geourjon, C. and Deleage, G. (1995) *Comput. Appl. Biosci.* 11, 681–684.
- [9] Kyte, J. and Doolittle, R.F. (1982) *J. Mol. Biol.* 157, 105–132.
- [10] Downie, B., Dirk, L.M., Hadfield, K.A., Wilkins, T.A., Bennett, A.B. and Bradford, K.J. (1998) *Anal. Biochem.* 264, 149–157.
- [11] Scognamiglio, M.A., Ciardiello, M.A., Tamburrini, M., Carratore, V., Rausch, T. and Camardella, L. (2003) *J. Protein Chem.* 22, 363–369.
- [12] Toubart, P., Desiderio, A., Salvi, G., Cervone, F., Daroda, L., De Lorenzo, G., Bergmann, C., Darvill, A.G. and Albersheim, P. (1992) *Plant J.* 2, 367–373.
- [13] Greiner, S., Rausch, T., Sonnewald, U. and Herbers, K. (1999) *Nat. Biotechnol.* 17, 708–711.
- [14] Greiner, S., Krausgrill, S. and Rausch, T. (1998) *Plant Physiol.* 116, 733–742.
- [15] Wolf, S., Grsic-Rausch, S., Rausch, T. and Greiner, S. (2003) *FEBS Lett.* 555, 551–555.
- [16] D'Avino, R., Camardella, L., Christensen, T.M.I.E., Giovane, A. and Servillo, L. (2003) *Proteins* 53, 830–839.
- [17] De Lorenzo, G., D'Ovidio, R. and Cervone, F. (2001) *Annu. Rev. Phytopathol.* 39, 313–335.
- [18] Dorokhov, Y.L., Makinen, K., Frolova, O.Y., Merits, A., Saarinen, J., Kalkkinen, N., Atabekov, J.G. and Saarma, M. (1999) *FEBS Lett.* 461, 223–228.
- [19] Chen, M.-H. and Citovsky, V. (2003) *Plant J.* 35, 386–392.
- [20] Le Cam, B., Massiot, P. and Rouxel, F. (1994) *Physiol. Mol. Plant Pathol.* 44, 187–198.
- [21] McMillan, G.P., Hedley, D., Fyffe, L. and Pérombelon, M.C.M. (1993) *Physiol. Mol. Plant Pathol.* 42, 279–289.