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Pectin methylesterase inhibitor cDNA from kiwi fruit

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Abstract We have newly isolated one partial pectin methylesterase inhibitor (PMEI) and two full-length cDNA clones from a kiwi fruit cDNA library. The two full-length cDNA clones, *Adpmei-1* and *Adpmei-2*, had an open reading frame of 185 amino acids, including a predicted signal peptide sequence necessary for localization in the cell-wall space. As the deduced amino acid sequence of the cloned fragment was almost same as the sequence of the previously purified PMEI protein (Camardella et al., Eur J Biochem 267:4561–4565), the clones were considered to be cDNAs encoding PMEI protein. Southern blot analysis indicated a low-copy number of the PMEI genes. Transgenic analysis of asparagus calli expressing a kiwi fruit PMEI gene driven by the CaMV 35S promoter demonstrated in vivo inhibition effects of PMEI on the endogenous pectin methylesterase (PME) activity. The relative expression levels of the PMEI genes in kiwi fruit, analyzed by competitive PCR, increased with the progression of fruit maturation. Given that PME activity also showed its highest level at the fully ripened stage of maturation, the increase in PMEI expression may not indicate direct inhibitory effects on the PME activity and fruit maturation process.

Keywords cDNA library · Kiwi fruit · Pectin methylesterase · Pectin methylesterase inhibitor · Southern blot hybridization

Abbreviations PGA: Polygalacturonic acid ·
PME: Pectin methylesterase · PMEI: PME inhibitor

Introduction

Pectin is a major constituent of the primary cell wall and the middle lamella in higher plants and is primarily composed of polygalacturonic acid (PGA). The carboxyl groups at the C6 position of PGA are methyl-esterified to varying degrees among different plant tissues or cell types (Carpita and Gibeaut 1993). Pectin methylesterase (PME; EC 3.1.1.11) catalyzes the demethylation of esterified PGA to the free carboxyl group. It has been proposed that this demethylation causes changes in tissue firmness that are related to physiological phenomena such as cell growth and elongation, fruit maturation, and defense responses (Goldberg et al. 1989; McCann and Roberts 1994; Bordenave 1996; Micheli 2001).

Pectin methylesterase inhibitor (PMEI), which acts as a powerful inhibitor protein of PME enzymes of plant origin, has been reported in kiwi fruit (Balestrieri et al. 1990; Castaldo et al. 1991; Giovane et al. 1995). This protein competitively inhibits PME enzymes through the formation of a noncovalent 1:1 complex (Giovane et al. 1995), and it has been suggested that the physiological role of PMEI is the regulation of cell-wall degradation and fruit-ripening processes by interaction with PME enzymes (Camardella et al. 2000; Jiang et al. 2002). The application of PMEI proteins to food technology is potentially interesting because PME causes many commercially deleterious effects such as phase separation during juice production and changes in tissue firmness during food processing (Castaldo et al. 1991; Andersson et al. 1994). The production of genetically modified crops containing PMEI genes is also an attractive possibility for inactivating endogenous PME activity. Camardella et al. (2000) recently determined the amino acid sequence and basic structure of PMEI using protein purified from kiwi fruit; however, genetic information with respect to PMEI and its physiological role during plant development was not reported. We report here the cloning of the first PMEI cDNA from kiwi fruit and its characterization.

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Materials and methods

Plant material

Kiwi fruit (*Actinidia deliciosa* cv. Heyword) plants were used for PMEI gene isolation. The plants were grown under natural conditions in the experimental garden of the National Institute of Fruit Tree Science, Akitu, Hiroshima, Japan. Fruit samples for analyzing the expression of PME and PMEI were harvested at different stages from flowering in May to maturation in November. Samples taken 1 month after postharvest ripening at room temperature were used as the fully ripened fruit stage.

Asparagus embryogenic calli (*Asparagus officinalis*) used for transformation experiments were a gift of the Hiroshima Prefectural Agriculture Research Center. They were cultured in MS liquid medium (Murashige and Skoog 1962) supplemented with 10 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) as described by Kohmura et al. (1994).

Cloning of a PMEI cDNA fragment and sequencing

A PMEI cDNA fragment was cloned by the rapid amplification of cDNA 3' ends (3' RACE) method. Total RNA was obtained from fully ripened fruit tissue using the method of phenol-lithium chloride extraction and ethanol precipitation. mRNA was purified from total RNA with Oligotex-dT30 super (Takara Bio., Japan). Single-stranded cDNA was synthesized from 5 μ g of mRNA with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, N.J.) using an oligo-dT-adapter primer (5'-GTTCTAG-ATCGCGAGCGGCCGCCCTTTT-3'), which includes a *Xba*I-*Nru*I-*Not*I adapter sequence. The synthesized cDNA was then amplified by PCR with a sense *Xba*I-*Nru*I-*Not*I adapter primer (5'-GTTCTAGATCGCGAGAAATCTGTCCGAAAAC-3') and an antisense degenerate primer (5'-GARATHGTGCC-IAARA-3', where R=A/G, Y=C/T, H=A/T/C, D=A/G/T). The primers were designed according to the amino acid sequence from Glu-7 to Thr-12 in the purified kiwi fruit PMEI protein (Camardella et al. 2000). PCR was carried out for 35 cycles of 30 s at 94°C (denaturation), 1 min at 41°C (annealing), and 1 min at 72°C (polymerization). The PCR products were subjected to 1.5% agarose gel electrophoresis, and DNA fragments of approximately 500 bp were excised from the gel and ligated to the alkaline-phosphatase-treated *Sma*I site in a pUC19 plasmid using a BKL kit (Takara Bio.).

The nucleotide sequence of the cloned fragment was determined by the dideoxy chain termination method using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) and a DNA autosequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems).

Cloning of full-length PMEI cDNAs

Two kinds of cDNA library were made by using mRNAs extracted from shoot tips and fully ripened fruits. cDNA was synthesized from 2 μ g of mRNA with a SuperScript Lambda System for cDNA Synthesis and a λ Cloning kit (GibcoBRL, Gaithersburg, Md.), ligated into λ ZIPLox phage vectors, and then packaged by using a Gigapack III Gold packaging extract (Stratagene, La Jolla, Calif.). The phages were transfected into *Escherichia coli* strain Y1090. Approximately 7.65×10^5 plaques in a library were blotted onto Hybond N⁺ nylon membranes (Amersham, UK). Probe labeling, plaque hybridization, and signal detection were carried out with a CDP-Star kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). After three screenings, positive cDNA clones were excised from λ ZIPLox phage vectors and inserted into pZL1 plasmids (Amersham Pharmacia Biotech).

Southern blot hybridization

Total DNA was isolated from the shoot apex and young leaves of kiwi fruit by the CTAB method (Murray and Thompson 1980), digested with restriction enzymes *Eco*RV, *Eco*RI, *Hinc*II *Hind*III, and *Xba*I (NEB, Beverly, Mass.), and subjected to Southern blot hybridization (Southern 1975). A 521-bp *Bam*HI-*Sac*I fragment of the PMEI cDNA clone obtained by the 3' RACE method was used as a probe. DNA probe labeling, hybridization, and signal detection were the same as those used in plaque hybridization.

Competitive reverse transcriptase-PCR

Total RNA was extracted from fruit tissues at five different stages during maturation by the phenol-lithium chloride extraction method and ethanol precipitation. mRNA was purified from total RNA with an Oligotex-dT30 super column (Takara Bio.). First-strand cDNA was synthesized by Superscript II reverse transcriptase (GibcoBRL) for 40 min at 50°C. The cDNA was then amplified by PCR with one denaturation cycle of 15 min at 95°C and 35 cycles of 55 s at 95°C, 1 min at 57°C, and 1 min at 72°C. One-microliter aliquots of the 50 μ l mRNA reaction mixture plus 1 μ l of each concentration of RNA competitor and sense (5'-GAAATCTGTCCCAAACTCG-3') and antisense (5'-TGAGTTGGTATATTCGGTG-3') primers were subjected to PCR using a one-step RT-PCR kit (QIAGEN, Valencia, Calif.). A gradient series ranging from 10^4 to 10^8 copies of RNA competitor was used for the analysis. The RNA competitor of the PMEI gene was designed to amplify cDNA fragments that were 72 bp shorter than the endogenous PMEI cDNA. The RNA competitor was obtained by in vitro transcription of the DNA competitor.

The DNA and RNA competitors were prepared using a Competitive DNA Construction kit (Takara Bio.) and an RNA Transcription kit (Takara Bio.), respectively, according to the manufacturer's protocols.

PME enzyme activity

PME enzyme activity was determined titrimetrically by monitoring increases in the number of free carboxylic groups (Van Buren et al. 1962). For enzyme extraction, the same fruit tissues that were used for competitive RT-PCR analysis were used. The carboxylic groups released by the extracted enzyme from 0.5% *Citrus* pectin in the presence of 0.125% NaCl were titrated against 0.02 N NaOH at pH 7.5 by using an automatic titrator (COM-900, Hiranuma).

Production and analysis of transgenic asparagus calli

A 577-bp PMEI cDNA fragment amplified by PCR using the *Bsr*GI-adapter primer (5'-CGGGTGTACTAGGGGGATCCAGG-3') and the *Sal*I-adapter primer (5'-ATTCGTCGACATGGCCTTTTCC-3') was digested with the *Bsr*GI and *Sal*I restriction enzymes and then ligated to the *Bsr*GI-*Sal*I site of the 3.4-kb plasmid CaMV 35S-sGFP-NOS3' (Chiu et al. 1996) lacking the sGFP coding sequence. The resulting plasmid was designated p35S-PMEI. Both of the p35S-PMEI and pCH plasmids, which harbored the hygromycin phosphotransferase (*hpt*) gene (Matsuki et al. 1989), or pCH alone was bombarded with gold particles on asparagus calli using the BIOLISTIC PDS-1000/He system (Bio-Rad, Hercules, Calif.) as described previously (Irifune et al. 2003). Following the bombardment, asparagus calli, still on their filter paper, were recultured on MS medium supplemented with 10 μ M 2,4-D and 0.8% agar for another 3 days. They were then transferred onto new MS medium containing 100 ppm hygromycin, 10 μ M 2,4-D, and 0.8% agar in a petri dish and subcultured every 3 weeks onto fresh hygromycin-containing medium under continuous light at 24°C for up to 3 months. DNA was extracted from hygromycin-resistant calli using the CTAB method (Murray and Thompson 1980). Randomly chosen transformed asparagus calli carrying both

the PME1 cDNA and the *hpt* gene, confirmed by PCR amplification, were analyzed for PME1 gene expression by the RT-PCR method using the primers sense 5'-GTTCTAGATCGCGAGAA-ATCTGTCCGAAAAC-3' and antisense 5'-TGAGTTGGTAT-ATTCCGTG-3'. PME enzyme activity was determined by the method described above.

Results and discussion

A 521-bp cDNA fragment amplified by the 3' RACE using 3' oligo dT and 5' degenerate primers was cloned. Except for three amino acid positions, the amino acid sequence deduced for the cloned fragment was the same as that determined for the previously purified PME1 protein (Camardella et al. 2000). Consequently, we considered the 521-bp cDNA fragment to be a cDNA clone encoding PME1 protein and named it *Adpmei-3* (accession no. AB091090). The cloned *Adpmei-3* fragment was then used as a probe to isolate the full-length PME1 cDNAs from kiwi fruit cDNA libraries.

Two kinds of cDNA library were constructed using RNA samples from the shoot tips and from fully ripened fruit tissues. After three screenings, four positive plaques were isolated from the fully ripened fruit cDNA library. Cloned fragments were excised from the λ ZIPLox phage vector and inserted into pZL1 plasmids (Amersham Pharmacia). No positive plaques were isolated from the shoot-tip cDNA library.

The nucleotide sequences of the four clones isolated from the fully ripened fruit cDNA library were determined. One of the four clones was 803 bp (*Adpmei-1*, accession no. AB091088), whereas the other three were 709 bp and shared the same nucleotide sequence (*Adpmei-2*, accession no. AB091089). Figure 1 shows the nucleotide sequence of *Adpmei-1*, the deduced open reading

frames (ORFs) of *Adpmei-1* and *Adpmei-2*, and the partial ORF of *Adpmei-3*. Two or three amino acid positions varied among the three clones. Except for the N-terminal sequences (upstream of Glu-33 in *Adpmei-1* and *Adpmei-2*), the deduced amino acid sequences of the three clones were consistent with the sequence of the purified kiwi fruit protein determined previously (Camardella et al. 2000). Considering that PME1 interacts with PME, which is localized in the cell wall, it is probable that a signal peptide is required to localize PME1 proteins to the cell-wall space. We suggest that the N-terminal 32 amino acids of the deduced *Adpmei-1* and *Adpmei-2* sequences are a signal peptide because they show features typical of a signal peptide: that is, a stretch of several hydrophobic amino acids and two positively charged amino acids (Markovic and Jornavall 1992; Micheli 2001).

A comparison of the amino acid sequence of *Adpmei-1* with those of other proteins in the database showed that *Adpmei-1* has a similarity to plant invertase inhibitors from *Platanus × acerifolia* (accession no. AJ427413), *Arabidopsis thaliana* (Y12807), and *Nicotiana tabacum* (T03393), with approximately 30% identity. In particular, the positions of four cysteine residues in the amino acid sequence are conserved among these proteins. Camardella et al. (2000) described a structural motif which forms two disulfide bridges. This motif is common among PME1 and plant invertase inhibitor proteins and acts as an inhibitor of enzymes involved in sugar metabolism. Raikhel et al. (1993) reported that structure-stabilizing disulfide bridges are a common feature of plant apoplastic proteins. A serine residue (corresponding to Ser-56 in *Adpmei-1* and *Adpmei-2*), which is a putative phosphorylation site, has also been found to be conserved among invertase inhibitor proteins (Jiang et al. 2002), although it has not been

Fig. 1 Nucleotide sequence of *Adpmei-1* and deduced amino acid sequences of *Adpmei-1*, *Adpmei-2*, and *Adpmei-3*. Identical amino acids are represented as dots (.). Arrows indicate the primers used for competitive RT-PCR amplification. An arrowhead above the sequence indicates the predicted cleavage site of the signal peptide estimated from the mature PME1 proteins from kiwi fruit (Camardella et al. 2000)

	GAGAAATTAAATTAATAAAAAAAGCAGTTTAAAGTGAAATAAGACTAGCTAGCA	55
<i>Adpmei-1</i>	ATGGCCTTTTCCTATTGTAGCTCTTCCTGTTTCTTCTTTACTTGTCATTCTATCATTAGCCCATTTGTC	130
ADPMEI-1	M A F S Y C S S S L F V S L L L V I L F I S P L S	25
ADPMEI-2	M A F S Y C S S S L F V S L L L V I L F I S P L S	25
<i>Adpmei-1</i>	CAAGGCCGAGCGTAAAGCAGAAACCATTTGATTAGCGAAATCTGTCCCAAACTCGCAATCTCTCTGTGC	205
ADPMEI-1	Q R P S V K A E N H L I S E I C P K T R N P S L C	50
ADPMEI-2	Q R P S V K A E N H L I S E I C P K T R N P S L C	50
ADPMEI-3	Q R P S V K A E N H L I S E I C P K T R N P S L C	12
<i>Adpmei-1</i>	CTGCAGGCTTTAGATCCGATCCTCGCTCAGCTAGTAAAGATCTTAAAGGCCCTTGGCCAGTTCTCTATCGACATA	280
ADPMEI-1	L Q A L E S D P R S A S K D L K G L G Q F S I D I	75
ADPMEI-2	L Q A L E S D P R S A S K D L K G L G Q F S I D I	75
ADPMEI-3	L Q A L E S D P R S A S K D L K G L G Q F S I D I	37
<i>Adpmei-1</i>	GCTCAGGCCAGTGCTAAACAAACCTCCAAATCATTCGCTCGCTTACAAACCAAGCAACTGACCCTAAACTCAA	355
ADPMEI-1	A Q A S A K Q T S K I I A S L T N Q A T D P K L K	100
ADPMEI-2	A Q A S A K Q T S K I I A S L T N Q A T D P K L K	100
ADPMEI-3	A Q A S A K Q T S K I I A S L T N Q A T D P K L K	62
<i>Adpmei-1</i>	GGTCGATACGAGACTTGCTCAGAAATATGCTGATGCGATCGATAGCCTTGGACAAGCCAAGCAGTTTGTGACA	430
ADPMEI-1	G R Y E T C S S E N Y A D A I D S L G Q A K Q F L T	125
ADPMEI-2	G R Y E T C S S E N Y A D A I D S L G Q A K Q F L T	125
ADPMEI-3	G R Y E T C S S E N Y A D A I D S L G Q A K Q F L T	87
<i>Adpmei-1</i>	TCCGGAGACTATAATAGCCTAAACATTACGCTTCCGCTGCCTTTGATGGGGCTGGAACCTGTGAAGATAGTTT	505
ADPMEI-1	S G D Y N S L N I Y A S A A F D G A G T C E D S F	150
ADPMEI-2	S G D Y N S L N I Y A S A A F D G A G T C E D S F	150
ADPMEI-3	S G D Y N S L N I Y A S A A F D G A G T C E D S F	112
<i>Adpmei-1</i>	GAAGGACCACCGAATATACCAACTCAACTCCCAAGCAGATCTGAAGTTGGAAGACCTTTGTGATATCGTTCTG	580
ADPMEI-1	E G P P N I P T Q L H Q A D L K L E D L C D I V L	175
ADPMEI-2	E G P P N I P T Q L H Q A D L K L E D L C D I V L	175
ADPMEI-3	E G P P N I P T Q L H Q A D L K L E D L C D I V L	137
<i>Adpmei-1</i>	GTTATATCTAATCTTTTGCCTGGATCAAAATAGTTTGTGTCATTGATTATGTCCAGTTGTGGCTACAGCTGATCA	655
ADPMEI-1	V I S N L L P G S K	185
ADPMEI-2	V I S N L L P G S K	185
ADPMEI-3	V I S N L L P G S K	162
<i>Adpmei-1</i>	ATAAATGAATTCCTCCATGCTTATAGTTACGAGCACTTATATTGGCATTAAAGCTCCTGATTTGGTACTCTGA	730
<i>Adpmei-1</i>	AGTTGTAACATAAACTAAATAACCGGTATTACGCCGAGGTACCTATATCTTTAATTCATGCATTTATGATC	803

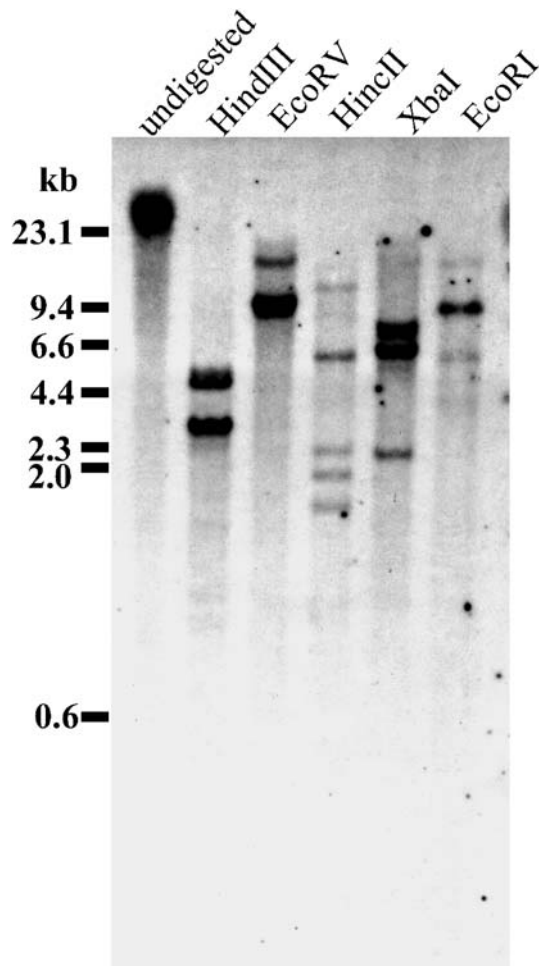


Fig. 2 Southern blot hybridization of total DNA from kiwi fruit. Ten micrograms each of undigested and *HincII*-, *EcoRV*-, *HindIII*-, *XbaI*-, and *EcoRI*-digested total DNA from the shoot apex plus young leaves was applied to each lane, separated by electrophoresis, and analyzed as described previously (Southern 1975). Molecular weight markers derived from *HindIII*-digested lambda DNA are shown on the left

demonstrated that phosphorylation functions as an activation switch in these proteins.

To determine the number of PMEI genes in kiwi fruit, we digested total DNA with different restriction enzymes and subjected it to Southern blot hybridization (Southern 1975). The 521-bp PMEI cDNA fragment (*Adpmei-3*) was used as a probe, and the results are shown in Fig. 2. Three to five hybridization bands were detected in each lane of the digested DNA. Given that there is only one recognition site for the *EcoRI* and *EcoRV* restriction enzymes in the probe and that there are no recognition sites for the other enzymes, it is difficult to accurately determine the number of the PMEI genes because of the inconsistency of the band numbers observed in each lane of digested DNA. However, it seems that the kiwi fruit genome has three or more PMEI genes, taking into account the three different PMEI cDNA clones, *Adpmei-1*, *Adpmei-2*, and *Adpmei-3*, that were isolated from an mRNA pool derived from ripe fruit.

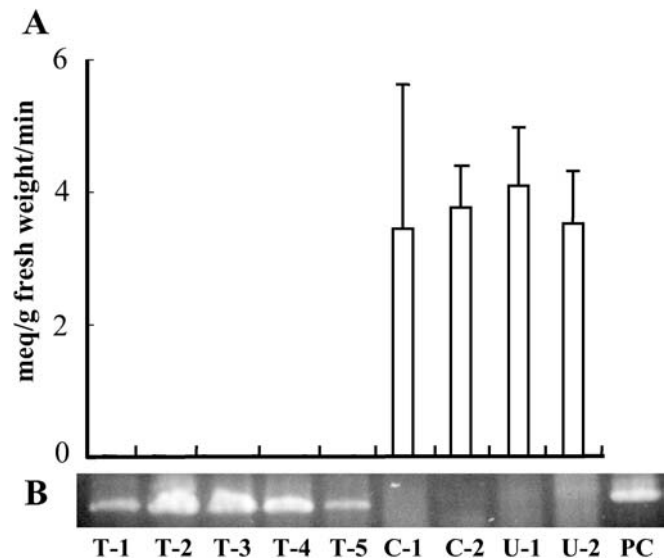


Fig. 3 PME enzyme activity (a) and agarose gel images (b) of the RT-PCR products in transformed and untransformed asparagus calli. *T-1*–*T-5* Clone numbers of the transformed calli carrying the kiwi fruit PMEI and *hpt* genes, *C-1*, *C-2* clone numbers of the transformed calli carrying the *hpt* gene alone, *U-1*, *U-2* clone number of untransformed calli, *PC* a positive control of plasmid p35S-PMEI. Vertical lines indicate standard deviations

Almost all plant PME enzymes are present as multiple isoforms and can be separated into at least two types on the basis of their gene expression pattern (Bordenave 1996; Micheli et al. 1998). Thus, one PME group shows ubiquitous expression, whereas another is expressed specifically during fruit ripening, pollen microsporogenesis, or stem elongation (Mu et al. 1994; Gaffe et al. 1997; Guglielmino et al. 1997; Micheli et al. 1998). In fact, 12 PME genes have been isolated from the *Arabidopsis* genome (Micheli et al. 1998), and more than ten independent PME or putative PME cDNA clones from tomato are entered in the database. However, it is estimated that there are fewer PMEI genes than PME genes in kiwi fruit. These few PMEI proteins are believed to have inhibitory effects on many different types of PME enzyme activity. In fact, extracted PMEI proteins from kiwi fruit show inhibitory activity in vitro against PME enzymes from various vegetable sources (Balestrieri et al. 1990).

To verify the in vivo inhibitory effects of PMEI function on the endogenous PME, we used particle gun bombardment to produce transgenic asparagus calli expressing kiwi fruit PMEI. Asparagus cells were chosen because of the inhibitory effects of kiwi fruit PMEI against monocot PME. Five randomly chosen transformed clones carrying the kiwi fruit PMEI and *hpt* genes, two transformed clones carrying the *hpt* gene alone, and two untransformed clones were used for our analysis of both PME enzyme activity and transcription levels of mRNA. No obvious PME activity levels were detected in any of the PMEI-transformed calli clones, while low but clear activity was detected in the control calli (Fig. 3a). With respect to the transcription levels of PMEI mRNA, the

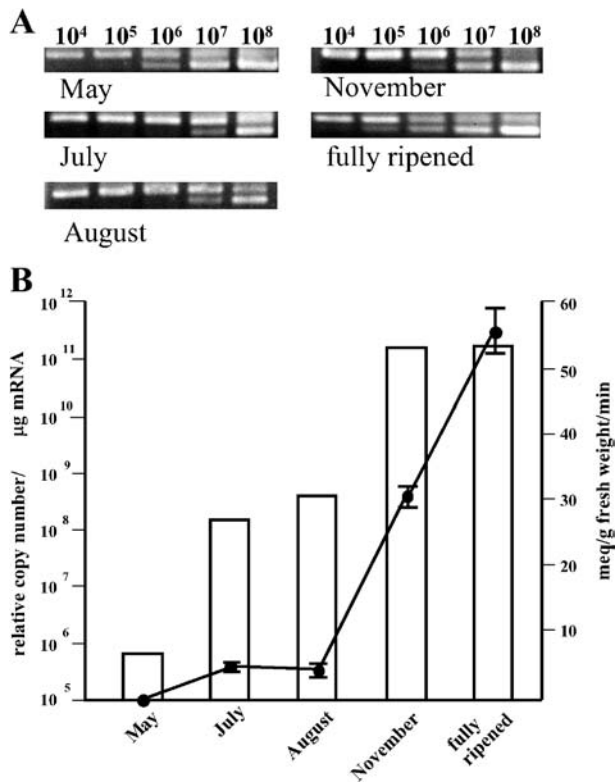


Fig. 4 Agarose gel images of the competitive RT-PCR products (a) and the relative copy number of PMEI genes and PME enzyme activity (b) during fruit maturation. **a** The following mRNA samples were used: 1.10 μ g in May, 0.37 μ g in July, 0.08 μ g in August, 0.39×10^{-4} μ g in November, 0.36×10^{-6} μ g in fully ripened fruit. Upper and lower bands represent amplified PMEI cDNA and competitor cDNA, respectively. **b** White bars indicate the relative copy number of PMEI mRNA calculated from the results shown in **a**. Line graph represents PME enzyme activity, vertical lines indicate standard deviations

predicted 362-bp amplified bands were obtained only in the lanes of mRNAs from the PMEI-transformed calli (Fig. 3b). These data suggest *in vivo* inhibitory effects of kiwi fruit PMEI on the endogenous PME activity in the transformed asparagus cells.

To analyze the expression of the PMEI genes in kiwi fruit, we determined the PMEI mRNA transcription levels using RT-PCR with an RNA competitor. As a preliminary step, we applied RT-PCR to observe the transcription levels of the PMEI genes in several kiwi tissues, including the root, young shoot, leaf, flower, and fully ripened fruits. The transcription levels of PMEI mRNA were very low or undetectable in each tissue except for the fully ripened fruit (data not shown). Therefore, competitive RT-PCR was performed with tissue samples taken at different maturation stages from flowering to the fully ripened fruit: that is, flowers in May, young fruit in both July and August, mature fruit in November, and fully ripened fruit at 1 month after postharvest ripening.

Figure 4 shows the agarose gel images of the competitive PCR products (Fig. 4a) and the relative expression levels of the PMEI genes and PME activity (Fig. 4b) during fruit maturation. Both the relative expression level

of the PMEI genes and PME activity increased along with the progression of fruit maturation. The expression level of PMEI increased markedly at the young fruit stages (from May to August), at which point the transcript level was 10²-fold higher than that at the flowering stage (Fig. 4b), whereas the PME activity only increased slightly. The expression level of PMEI increased markedly again during the maturation stage (from August to November), at which point the transcript level was 10³-fold higher than that at the young fruit stage (Fig. 4b); simultaneously, PME activity increased markedly and continued to increase during postharvest ripening. An increase in PME enzyme activity corresponding to fruit softening has been reported in banana (Hultin and Levine 1965), tomato (Koch and Nevins 1989; Harriman et al. 1991), highbush blueberries (Proctor and Miesle 1991), and papaya (Catutani and Lourenco 1982). In kiwi fruit, PME activity has been shown to increase for 15 days during postharvest ripening at 20°C (Matsumoto and Takano 1993). Indeed, postharvest ripening is a commercially important treatment for fruit softening in kiwi fruit. It seems that an apparent increase in PME activity affects kiwi fruit softening during postharvest ripening.

It would appear to be an anomalous situation that there is successively higher expression levels of PMEI at the postharvest ripening stage even though PME activity is also at a peak and the fruits are becoming fully softened. This may suggest post-translational controls of the PMEI protein. Giovane et al. (1995) has reported that PMEI is synthesized as an inactive precursor of higher molecular mass and subsequently transformed into the active protein during the fruit ripening process. At an unripe stage, neither an active PMEI band (28 kDa) was recognized by sodium dodecyl sulfate/polyacrylamide gel electrophoresis by the antibodies against PMEI nor was inhibitory activity detected, although a band of a higher molecular mass (30 kDa) was recognized. In contrast, during the ripening stage, active PMEI bands were observed and PMEI activity became detectable (Giovane et al. 1995). On the other hand, high expression levels of PMEI mRNA before ripening (approx. November) may indicate an accumulation of the inactive form of PMEI. However, the mechanism for inducing PMEI activity from an inactive form is not known. Another possibility is that apart from the endogenous PME inhibition, PMEI has another physiological role, for example, as a defense-related protein, which would also require the common disulfide bridge structure (Balestrieri et al. 1990). Raikhel et al. (1993) indicated that disulfide bridges were a common feature of other plant apoplastic proteins, such as peroxidases and the chitin-binding domain of several defense-related proteins. It is unclear whether the increase in PMEI expression translates directly into an increase in inhibitory activity against PME enzymes or not.

We report here for the first time isolation of the PMEI cDNA and its expression levels at different stages of fruit maturation. Because the identification of PMEI proteins to date has been reported only from a kiwi fruit (Giovane et al. 1995; Camardella et al. 2000) and a jelly fig (*Ficus*

awkeotsang) (Jiang et al. 2002), the analysis of the kiwi *Adpmei* nucleotide sequences will provide information that will aid in the isolation of PMEI genes from other plant species using a genetic approach. We have demonstrated in vivo inhibitory effects of kiwi fruit PMEI on the endogenous PME activity in the transformed asparagus cells. However, the biochemical effect of PMEI proteins on cell-wall metabolism and their physiological role during plant development are still unclear. To identify the physiological function of PMEI and to examine the possible utilization of PMEI in the food industry, we are now investigating the development of transgenic PMEI plants using sense and antisense *Adpmei* cDNAs.

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