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Characterization and Functional Expression of a Ubiquitously Expressed Tomato Pectin Methylesterase[1]

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Pectin methylesterase (PME), a ubiquitous enzyme in plants, de-esterifies the methoxylated pectin in the plant cell wall. We have characterized a PME gene (designated as *pmeu1*) from tomato (*Lycopersicon esculentum*) with an expression that is higher in younger root, leaf, and fruit tissues than in older tissues. Hypocotyls and epicotyls show higher accumulation of *pmeu1* transcripts compared with cotyledons. *pmeu1* represents a single-copy gene in the tomato genome. Comparison of the deduced amino acid sequence of *pmeu1* with other *PME* homologs showed that the N-terminal halves are highly variable, and the C-terminal halves are relatively conserved in plant PMEs. Constitutive expression of a fruit-specific PME antisense gene does not affect the level of *pmeu1* transcripts in vegetative tissues but does lower the level of PMEU1 mRNA in developing tomato fruits. These results suggest that there exists developmentally regulated silencing of *pmeu1* by a heterologous PME antisense gene. Expression of *pmeu1* in tobacco (*Nicotiana tabacum*) under the control of the cauliflower mosaic virus 35S promoter caused up to a 4-fold increase in PME specific activity that was correlated with the accumulation of PMEU1 mRNA. In vitro transcription-translation analyses show that *pmeu1* encodes a 64-kD polypeptide, whereas transgenic tobacco plants expressing *pmeu1* accumulate a new 37-kD polypeptide, suggesting extensive posttranslational processing of PMEU1. These results are the first evidence, to our knowledge, of the functional characterization of a PME gene and the extensive modification of the encoded polypeptide.

The plant extracellular matrix is an intricate structure that is involved in cell shape, growth, and development, and interaction with the environment, including biotic and abiotic stresses. Most working models of the primary cell wall depict it as a network of cellulose and cross-linking glycans embedded in the pectic substances of the gel matrix and strengthened by structural proteins and other components (Carpita and Gibeaut, 1993). During the last 5 years, significant progress has been made in revealing the fine structure of cell wall polysaccharides and the roles of cell wall-associated enzymes, including glucanases, xyloglucan-endoglycosyltransferases, expansins (a family of proteins that induce cell wall extension in isolated cell walls), and other structural proteins (for review, see Carpita et al., 1996).

Pectic substances are a major component of the primary cell wall and middle lamella of dicotyledons and are composed primarily of polygalacturonic acid and rhamnogalacturonan I. Rhamnogalacturonan II, a highly heterogeneous polymer, is another pectic compound of the plant cell wall but is too scarce to be a major structural component (for review, see Carpita and Gibeaut, 1993). Polygalacturonic acids represent helical homopolymers of (1→ 4) α-D-galactopyranosyluronic acid with a varying degree of methylesterified carboxylic groups, whereas rhamnogalacturonans I are heteropolymers of (1→ 2) α-L-rhamnosyl-(1→ 4) α-D-galacturonic acid disaccharide units. Pectins have been suggested to be highly methylesterified when synthesized and secreted to the cell wall before some of the methyl esters are cleaved (Jarvis, 1984)

PME catalyzes the demethoxylation of pectins and is considered to be responsible for chemical modifications of pectin embedded in the plant's primary cell wall matrix. Although PME is known to be a ubiquitous enzyme in the plant kingdom (for review, see Sajjaanantakul and Pitifer, 1991), and a diversity in structure and distribution of esterified and nonesterified pectins in the plant cell wall has been revealed using several novel approaches (Knox et al., 1990; Liners and Van Cutsem, 1992; Roy et al., 1994), the function of pectin modification in plant growth and development remains to be elucidated. Emerging evidence indicates that pectins are involved in determining cell wall porosity, providing charged surfaces in the cell wall to modulate the pH and ion balance, and acting as signaling molecules during association with pathogens and insects (Shedletzky et al., 1990; Grignon and Sentenac, 1991; Bellincampi et al., 1993). Functional replacement of the microfibrillar framework with a tightly cross-linked pectin matrix in the cell wall has been demonstrated for tomato (*Lycopersicon esculentum*) cells adapted to grow in the presence of a cellulose biosynthesis inhibitor (Shedletzky et al., 1990). PME has been suggested to be involved in cell wall growth, extensibility, and regeneration, in the separation of root border cells from the root cap, and in the formation of abscission zones and textural changes in ripening fruit (Sexton and Roberts, 1982; Lamport, 1986; Shea et al., 1989; Nari et al., 1991; Stephenson and Hawes, 1994; Tieman and Handa, 1994). However, despite these studies direct evi-

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Abbreviations: CaMV 35S, cauliflower mosaic virus 35S; ORF, open reading frame; PME, pectin methylesterase.

dence for PME involvement in plant growth and development is lacking.

Multiple isozymes of PME are present in different tissues of plants, including tomato (Gaffe et al., 1994). We have reported the presence of both fruit-specific and ubiquitously expressed isoforms of PME in tomato. Expression of the latter class of PME isoforms is not impaired by the constitutive expression of a fruit-specific PME antisense gene in vegetative and fruit tissues (Gaffe et al., 1994). Inhibition of fruit-specific PME gene expression by its antisense gene results in the loss of tissue integrity of fruit pericarp but does not affect the growth and development of the tomato plant (Tieman et al., 1992; Hall et al., 1993, Tieman and Handa, 1994). The fruit PME seems to regulate the cation-binding capacity and the cation selectivity in ripening tomato fruits (Tieman and Handa, 1994).

To elucidate the role of ubiquitously expressed PME isozyme(s), we have cloned a PME cDNA (designated *pmeu1*) from cDNA libraries made from leaf and root tissues (Gaffe et al., 1996). We report here that *pmeu1* is expressed in all tomato organs analyzed, and its transcript levels are not affected by the fruit-specific PME antisense gene in vegetative tissues. *pmeu1* is encoded by a unique, single-copy gene in the tomato genome. We further demonstrate, by expressing *pmeu1* cDNA under the control of the CaMV 35S promoter in transgenic tobacco (*Nicotiana tabacum*) plants, that it encodes an active PME enzyme.

MATERIALS AND METHODS

Tissue and Plant Preparation

Wild-type tomato plants (*Lycopersicon esculentum* cv Rutgers), transgenic tomato plants (3781^) expressing a fruit PME antisense gene (Tieman et al., 1992), and transgenic tobacco (*Nicotiana tabacum*) plants expressing *pmeu1* or the vector alone were grown in the greenhouse under standard conditions. Tomato fruits were obtained from plants grown in the field during the summers of 1995 and 1996 at a research farm. Flowers were tagged and fruits were harvested at different stages of development. Tissues were frozen in liquid N_2 and stored at −80°C until use.

Protein Extraction, Protein Analysis, and PME Activity

Plant tissues were ground in liquid N_2 and total proteins were extracted in an equal volume (w/v) of 2 M NaCl. PME enzymatic activity and total protein were determined as described previously (Gaffe et al., 1994).

RNA and DNA Extraction and Analysis

Total RNAs from tobacco and tomato roots, leaves, flower, stems, and seedlings were extracted according to the method of Chomczynski and Sacchi (1987). Fruit RNAs were extracted according to the method of Tieman et al. (1992). Fifteen micrograms of total RNA was size-fractionated on a 1.2% (w/v) agarose denaturing formaldehyde gel (Sambrook et al., 1989), blotted onto a Hybond-N membrane (Amersham), and hybridized with a ^{32}P-labeled probe (0.5–1 × 10^6 cpm mL^{-1} hybridization solution). DNA probes were labeled with [^{32}P]dCTP using a random-primer labeling kit (DECA-Prime I, Ambion, Austin, TX). Membranes were washed three times for 15 min in 2× SSC containing 0.1% SDS at room temperature and then washed three times more for 15 min in 1× SSC containing 0.1% SDS at 65°C for low-stringency conditions. For high-stringency conditions, an additional three 10-min washes in 0.1× SSC containing 0.1% SDS at 65°C were performed. Total plant genomic DNA was extracted as previously described (Tieman et al., 1992), digested with the indicated restriction endonucleases, electrophoresed, blotted, and hybridized to ^{32}P-labeled probe under the same conditions described above.

In Vitro Transcription-Translation of *pmeu1*

pmeu1 cloned in plasmid pBSKS (Stratagene) was digested with *Bam*HI. The linearized *pmeu1*-containing plasmid was transcribed using the Maxiscript kit and T3 polymerase (Ambion) and capped with m^7GpppG (Boehringer Mannheim). The resulting RNAs were translated using rabbit reticulocytes (Promega) in the presence of [^{35}S]Met (United States Biochemical-Amersham), according to the manufacturer's instructions. Radiolabeled products were analyzed on 10% SDS-PAGE (Laemmli, 1970). Gels were briefly stained with Coomassie brilliant blue, soaked in En^{3}Hance (DuPont), rinsed, and autoradiographed.

5′ Extension Analysis

Primer extension analysis to determine the transcription start site of *pmeu1* was performed according to the method of Raghothama et al. (1991) on total root RNA. The antisense primer (5′-CAACAGGTGTCATTTTGG-3′) was end-labeled with [γ^{32}P]dATP (Amersham) using polynucleotide kinase (United States Biochemical-Amersham). Primer-extended products were analyzed on a 6% DNA-sequencing gel using a sequencing reaction ladder.

Construction of a Chimeric *pmeu1* Gene and Tobacco Transformation

A 1920-bp *pmeu1* cDNA insert containing 12 bp from the 5′ untranslated region, the complete ORF, and the longest 3′ untranslated region were released after digestion with *Xho*I and *Xba*I and force-cloned into a *Xho*I and *Xba*I-digested pKYLX71 in the sense orientation. pKYLX71 is a derivative of pKYLX7 (Schardl et al., 1987). The resulting construct, pKYLXPMEU1, was mobilized into *Agrobacterium tumefaciens* plasmid LBA4404 after triparental mating using pRK2013 as a helper plasmid and rifampicin plus tetracycline as selectable markers (Tieman et al., 1992). Leaf discs from tobacco (cv Wisconsin 38) were transformed with either pKYLXPMEU1 or pKYLX71, and transformed plants were selected on kanamycin-containing media (Horsch et al., 1985).

RESULTS

Transcription Start Site and in Vitro Transcription-Translation of *pmeu1*

We have previously reported the nucleotide sequence of a PME cDNA homolog (designated *pmeu1*) isolated from cDNA libraries made from poly(A^+) RNAs isolated from tomato leaf and root tissues (Gaffe et al., 1996). The largest ORF in *pmeu1* cDNA encodes a 583-amino acid polypeptide with a deduced molecular mass of 64 kD. However, molecular masses of PME isoforms purified from different tomato tissues range between 23.8 and 42 kD (Harriman et al., 1991; Pressey and Woods, 1992; M.E. Tiznado, J. Gaffe, and A.K. Handa, unpublished results). To establish the fidelity of the observed largest ORF, transcription start site and in vitro transcription-translation analyses of *pmeu1* were performed. Figure 1A shows that the transcription start site for *pmeu1* is 79 bp from the putative translational codon for the largest ORF. Another ATG was located 6 bp from the determined transcription start site but was followed by an in-frame stop codon 18 bp from the transcription start site.

Figure 1B shows the SDS-PAGE analysis of the in vitro transcription-translation of *pmeu1* in the presence of [^{35}S]Met, as described in "Materials and Methods." Several polypeptides were obtained, ranging in size from 42 to 64 kD (Fig. 1B). The 64-kD polypeptide represents the expected polypeptide encoded by *pmeu1* (Gaffe et al., 1996). The identity of smaller-sized polypeptides is not clear but may represent partial translation products. We have isolated three classes of *pmeu1* cDNAs with polyadenylation occurring at the nucleotides 1918, 1994, and 2034 of *pmeu1* (data not shown). However, the consensus polyadenylation signal sequence at nucleotide 1897 was noted for only the first site. The AT enrichment in the remaining 3′ untranslated region may promote polyadenylation at other sites (Leuhrsen and Walbot, 1994). These results show that the *pmeu1* ORF encodes a polypeptide of 64 kD and that the translation start site is preceded by a 79-bp untranslated region.

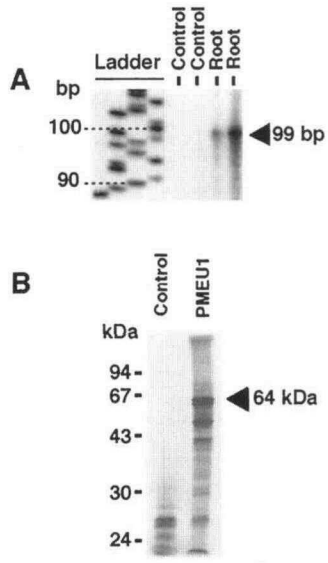

Figure 1. Transcription start-site (A) and in vitro transcription-translation (B) of *pmeu1*. A, Total root RNA was reverse-transcribed using the radiolabeled antisense oligonucleotide primer ^{32}P-CAACAGGTGTCATTTTGG and Moloney murine leukemia virus reverse-transcriptase. Products were separated on a 6% DNA-sequencing gel and autoradiographed. The transcription start site was deduced from a sequencing ladder reaction of a known plasmid. "Control" and "Root" represent the reverse transcription reactions in the absence and the presence of total root RNA, respectively. B, Autoradiograph of in vitro transcription-translation product of *pmeu1*. *pmeu1* cloned in pBSKS was in vitro transcribed, capped, and translated in the presence of [^{35}S]Met as described in the experimental procedures. Radiolabeled products were separated on a 10% SDS-PAGE gel and autoradiographed. "Control" and "PMEU1" represent the transcription-translation reaction products in the absence and presence of the *pmeu1* cDNA template, respectively.

Analysis of the Deduced Amino Acid Sequence of *pmeu1*

Comparison of the deduced amino acid sequence of *pmeu1* with other plant PME homologs showed that the 264 amino acid residues at the C-terminal end of *pmeu1* share significant similarity with other cloned PME homologs (Fig. 2). About 250 amino acid residues at the N-terminal end of various PME homologs (except PMEU1 and AT-PME2) share much less similarity with each other (Fig. 2). With 69% identity over the entire range of the deduced amino acid sequence, PMEU1 is highly similar to ATPME2 from *Arabidopsis thaliana* (Table I). The first 260 amino acid residues at the N terminus and 278 amino acid residues at the C terminus of PMEU1 show 69 and 77% identity with respective regions of ATPME2. All other plant PMEs show less than 20% identity with the 260 amino acid residues present at the N terminus of PMEU1 and between 44 to 60% identity with the 278 amino acid residues present at the C terminus of PMEU1 (Table I).

Chou-Fasman or Robson-Garnier two-dimensional structure analysis of the deduced amino acid sequences of plant PMEs, including *pMEU1* (Fig. 3A), show that the N-terminal halves of all PMEs are enriched in the α-helix structure with a low abundance of β-sheets, whereas the C-terminal halves comprised many β-sheets (data not shown). The N-terminal region of *pMEU1* contains a hydrophilic and a hydrophobic arrangement typical of a signal peptide with a predicted cleavage site at Ala-40 (Von Heijne, 1986; Figs. 2 and 3). The hydrophobic core of the putative signal peptide is flanked by five basic amino acid residues at the N side and several slightly acidic residues at the C side, suggesting an external localization of the C side (Von Heijne and Gavel, 1988). Similar putative signal peptides are encoded in several PME homolog cDNAs, including cDNAs from tomato (Hall et al., 1994; Turner et al., 1996) and peach (*Prunus persica*; Glover et al., 1996) fruits, *A. thaliana* (Richard et al., 1994, 1996), pea (*Pisum sativum*), and *Brassica napus* (Albani et al., 1991; Recourt et al., 1995), and might play a role in translocation of encoded polypeptides into the ER.

In addition to the putative signal peptide region, the PMEU1 has several additional strong hydrophobic regions (Fig. 3B). Similar hydrophobic regions are present in

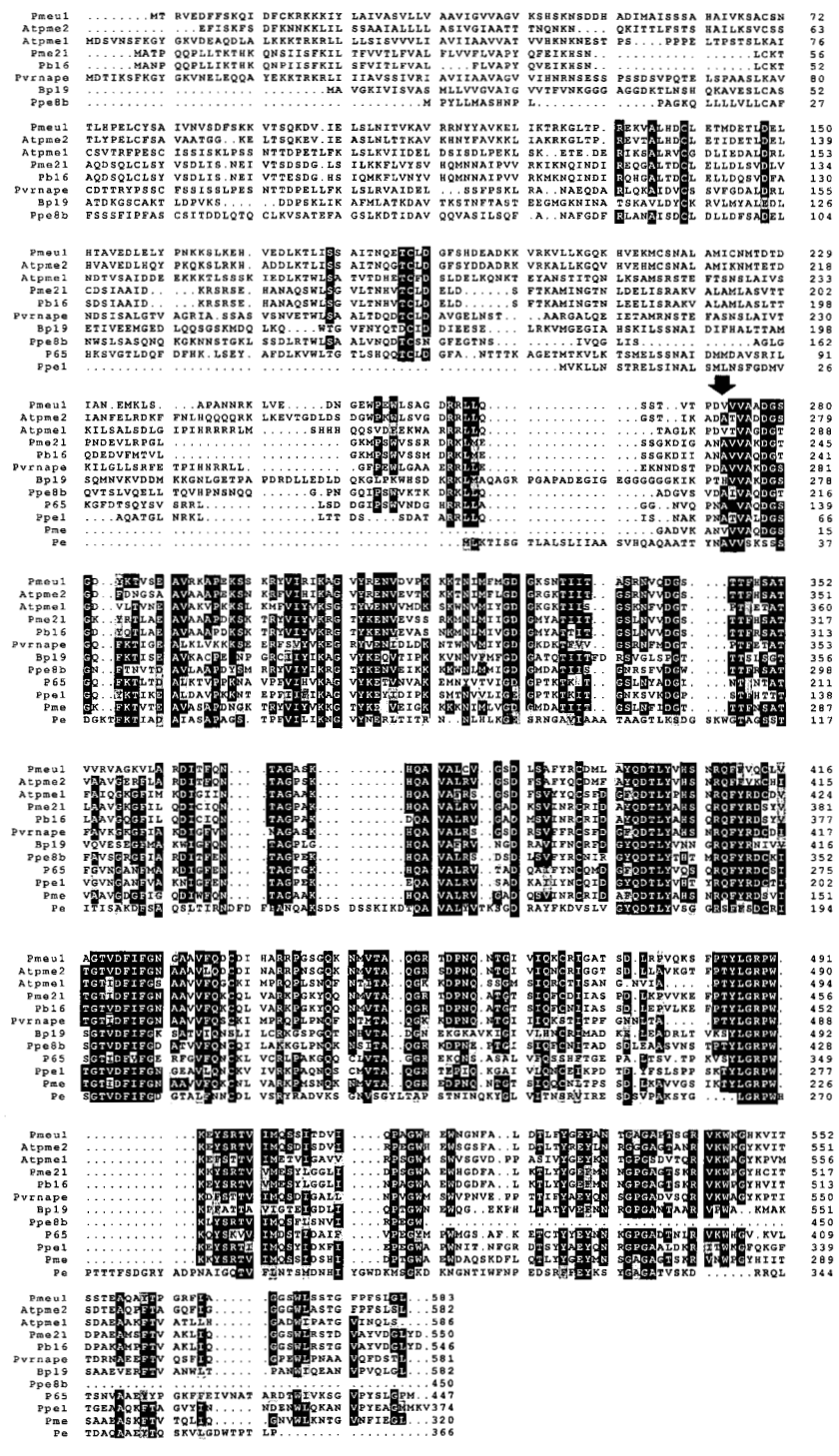

Figure 2. Alignment of the deduced amino acid sequences of PME genes. Alignment of deduced amino acid sequences was performed on PME homologs using GCG's Pileup program (Genetics Computer Group, Madison, WI). The arrow indicates the N terminus of a purified PME protein from tomato (Markovic and Jornvall, 1986) and defines the variable N terminus and the relatively conserved C-terminal domains of various PMEs. Also shown is the deduced amino acid sequence of a PME gene from the phytopathogenic bacterium *Erwinia chrysanthemi*. Shading corresponds to similar amino acid sequence in at least seven PME sequences. pmeu1, *pmeu1* from tomato plant (U49330); Pme21, *PME2.1* from tomato fruits (U50986); Pb16, *pB16* from tomato fruit (X74639); Atpme1, *ATPME1* from Arabidopsis young tissues (X81585); Atpme2, *ATPME2* Arabidopsis genomic clone (U25649); Pvrnape, *PvRNAPE* from pea pod (X85216); Bp19, *BP19* from *B. napus* pollen (X56195); Ppe1, *Ppe1* from petunia (*Petunia inflata*) pollen (L27101); Ppe8b, *PPE8B* from peach fruit (X95991); P65, *P65* from alfalfa (*Medicago sativa*) pollen (U28148); Pme, *PME* from mung bean (*Vigna radiata*) hypocotyl (X94443); Pe, *PE* from *E. chrysanthemi* (Y00549).

ATPME1 from Arabidopsis (Richard et al., 1994), PVRNAPE from pea (Recourt et al., 1995), Bp19 from *B. napus* (Albani et al., 1991), and pB16 from tomato fruit (Hall et al., 1994), but not in pB8 from tomato fruit (Hall et al., 1994), ATPME2 from Arabidopsis, or Ppe1 from petunia (Mu et al., 1994).

Analysis of the phylogeny was performed for all cloned PMEs, including a PME gene from *E. chrysanthemi* (Plastow, 1988), using the computer programs Pileup, Distances (the Kimura method), and Growtree (the neighbor-joining method) of the GCG package. Because of a high degree of variability observed in the N terminus half of the various PMEs, independent analyses of both the N- and C-terminal halves were performed. Figure 4 shows the phylogenic analysis based on the C-terminal conserved regions of various PMEs. Several distinct groups appeared, one contain-

Table I. *Sequence identities and similarities between PMEU1 and other PME cDNA*

Based on the N terminus of a PME purified from tomato fruits (Markovic and Jornvall, 1986) the deduced amino acid sequences of cloned PMEs were divided into N-terminal and C-terminal regions.

PME	Species	Deduced Polypeptide			N-Terminal Region			C-Terminal Region		
		AA[a]	S[b]	I[c]	AA	S	I	AA	S	I
			%			%			%	
PMEU1	*L. esculentum*	583	100	100	268	100	100	315	100	100
ATPME2	*A. thaliana*	582	76	69	268	72	60	314	79	77
pB16	*L. esculentum*	546	59	40	229	46	17	317	69	60
PME2.1	*L. esculentum*	550	58	40	233	46	19	317	69	58
PME	*V. radiata*	320	67	32	3	nd[d]	nd	317	68	58
ATPME1	*A. thaliana*	586	53	34	276	46	17	310	60	49
PvRNAPE	*P. vulgaris*	581	54	33	269	48	15	312	61	49
Bp19	*B. napus*	584	50	31	268	43	15	316	56	49
Ppe1	*P. inflata*	374	58	28	55	nd	nd	319	59	44
P65	*M. sativa*	447	56	29	127	32	12	318	59	44
PPE8B	*P. persicus*	450	51	30	204	44	14	246	60	44
PE	*E. chrysamthemi*	366	38	15	25	nd	nd	341	50	27

[a] AA, Amino acid residues used to determine S and I. [b] S, Percentage similarities with the corresponding region of PMEU1. [c] I, Percentage identities with the corresponding region of PMEU1. [d] nd, Not determined.

ing PMEU1 and the Arabidopsis ATPME2 and others containing the pollen PMEs, the tomato fruit PMEs, Arabidopsis ATPME1, and the *B. napus* PME.

Genomic Organization of *pmeu1*

Figure 5 shows the tomato genomic DNA-blot analysis using probes for *pmeu1* and a fruit-specific PME. Single *Eco*RI, *Sal*I, and *Xba*I fragments of 5.5, 10.3, and 13.7 kb, respectively, hybridized to *pmeu1*. *pmeu1* cDNA contains an internal *Hin*dIII site, and digestion of tomato genomic DNA with *Hin*dIII generated two hybridizing DNA fragments of 5.8 and 1.8 kb. A different and more complex hybridization pattern is obtained when the same blot is probed with a fruit PME cDNA (Fig. 5). These results indicate that *pmeu1* does not belong to the previously characterized fruit-specific PME gene family and that the tomato genome contains one gene homologous to the isolated *pmeu1* cDNA. Analysis of genomic DNA from other Solanacae species indicates that *pmeu1* homologs exist in potato (*Solanus tuberosum*) and tobacco (Fig. 5). The complex patterns for potato and tobacco genomic DNA are most likely due to the amphidiploid nature of these species.

Ubiquitous Expression of *pmeu1*

Figure 6 shows the RNA expression of *pmeu1* in different organs of seedlings and mature tomato plants. *pmeu1* tran-

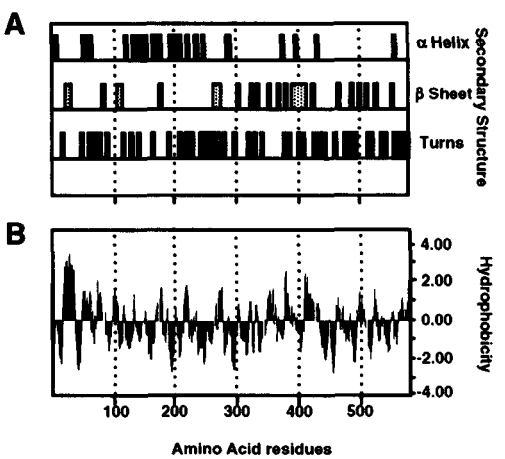

Figure 3. Secondary structure (A) and hydrophobicity plots (B) of the deduced amino acid sequence of *pmeu1*. Deduced amino acid sequence from *pmeu1* was analyzed with the program Mac Vector (Kodak) on a window of seven amino acid residues. A, Two-dimensional Chou-Fassman structure analysis for α-helix, β-sheet, and turn localization. B, Hydrophobicity analysis using the Kyte Doolittle algorithm.

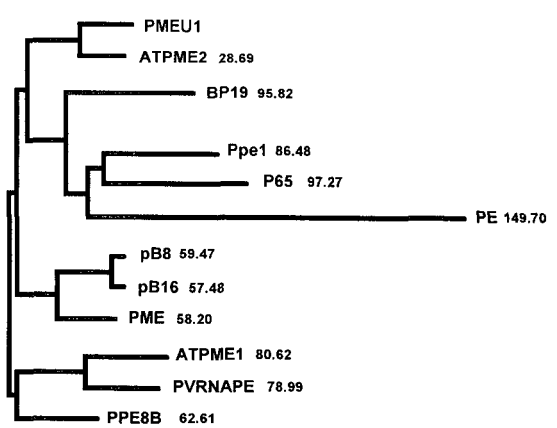

Figure 4. Evolutionary relatedness of *pmeu1* to other PMEs. Phylogenic analysis was based on the C-terminal conserved regions of different PME homologs defined in Figure 2. The unrooted tree was calculated using the programs Distances (Kimura method) and Growtree (neighboring joining method) of the GCG package and the *pmeu1* C-terminal region as the origin for the calculation. The numbers indicate substitutions per 100 amino acid residues.

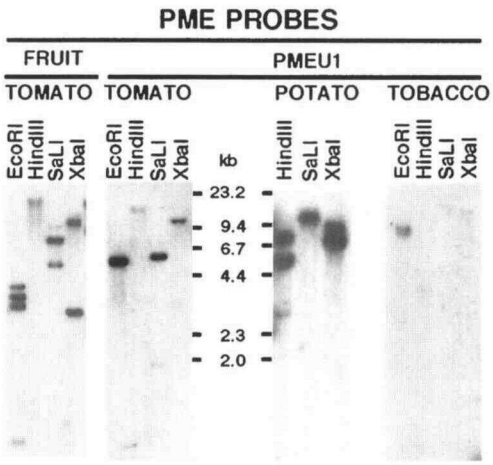

Figure 5. Genomic organization of *pmeu1*. Fifteen micrograms of tomato, potato, and tobacco genomic DNA were digested with the indicated restriction endonucleases and subjected to DNA gel-blot analysis. Radiolabeled insert from the 2.1-kb tomato fruit PME (Turner et al., 1996) and 1.7 kb from *pmeu1* cDNA clones were used as hybridization probes.

script was present in all of the tomato organs analyzed, but the levels of *pmeu1* mRNA were higher in younger tissues than in mature tissues. Young, expanding leaves showed approximately 2- to 3- fold higher levels of *pmeu1* mRNA levels than fully expanded leaves (Fig. 6A). A similar pattern was observed in roots from young and mature tomato plants. Higher levels of *pmeu1* RNA were also observed in the apex and flower tissues. Hypocotyl and epicotyl tissues showed higher levels of *pmeu1* transcript than cotyledon tissues (Fig. 6B). *pmeu1* was strongly expressed in young fruit, but its mRNA levels declined during the later stages of fruit development, with a significant decrease in ripening fruit (Fig. 6C). The pattern of *pmeu1* mRNA accumulation in developing tomato fruit differed from that of the fruit-specific PMEs characterized previously (Harriman et al., 1991; Hall et al., 1994). While the *pmeu1* mRNA levels declined, the fruit-specific PME mRNA levels increased during fruit development (Fig. 6C).

Expression of *pmeu1* was examined in different organs of transgenic tomato expressing a fruit-specific PME antisense gene under the control of the CaMV 35S promoter (Tieman et al., 1992). The accumulation of *pmeu1* RNA was not affected in vegetative tissues but declined earlier in developing fruit from transgenic plants compared with wild-type tomato plants (Fig. 6C). *pmeu1* transcripts were not detectable in 35-d or older transgenic fruits, but accumulation of the fruit-specific PME transcripts was completely abolished by the expression of the fruit-specific PME antisense gene (Fig. 6C; Tieman et al., 1992). *pmeu1* and the fruit PME genes do not cross-hybridize with each other under these experimental conditions (Fig. 6D). Taken together, these data indicate that fruit PME antisense gene expression has an inhibitory effect on the accumulation of *pmeu1* mRNA in developing transgenic fruits but not in the vegetative tissues.

pmeu1 Encodes an Active PME Enzyme in Transgenic Tobacco Plants

We have expressed *pmeu1* under the control of the CaMV 35S promoter in transgenic tobacco plants to establish that it encodes an active PME enzyme. A *pmeu1* chimeric gene construct (pKYLXPMEU1, Fig. 7A) was created by introducing the *pmeu1* cDNA into pKYLX71, an *A. tumefaciens*-based transformation vector (Schardl et al., 1987). This chimeric *pmeu1* gene construct and vector (pKYLX71) were used to create transgenic tobacco plants. Several independent transgenic tobacco plants transformed with pKYLXPMEU1 and pKYLX71 were characterized for expression of the *pmeu1* chimeric gene.

Total RNA from young, expanding leaves of several transgenic tobacco plants transformed with pKYLXPMEU1 contained a 2.3-kb RNA species that strongly hybridized with a *pmeu1*-specific probe (Fig. 7C). This RNA was not detected in transgenic plants transformed with pKYLX71 alone (Fig. 7C). When probed with a *pmeu1* probe representing the conserved regions of plant PMEs, weak hybridization to an RNA species of about 2 kb was observed from plants transformed with either pKYLXPMEU1 or

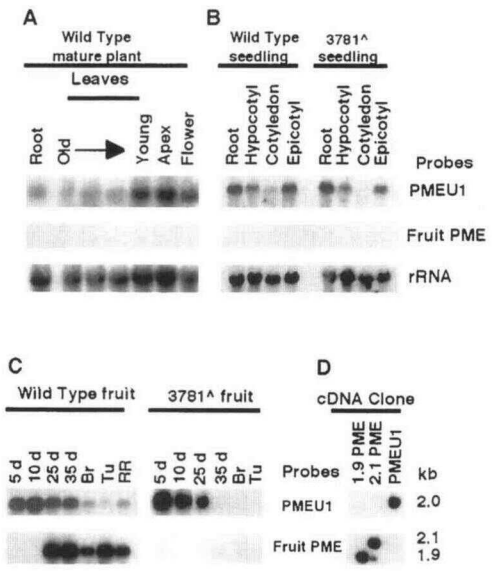

Figure 6. Expression of *pmeu1* and a fruit PME gene in young and mature tomato plant organs and developing fruit pericarp of wild-type and transgenic plants expressing a fruit-specific antisense gene. Total RNA (15 μg) from various tissues of wild-type tomato and transgenic (3781^) tomato plants expressing a fruit PME antisense under the control of the CaMV 35S promoter were separated on agarose gels, blotted, and hybridized with either a *pmeu1* or a fruit PME cDNA probe. The *pmeu1* probe represents 0.8 kb from the 5′ region and the fruit *PME* probe represents the 2.1-kb PME cDNA insert. Accumulation of *pmeu1* and fruit PME transcripts in A, root and leaves representing the bottom (old) to the top (young) of tomato plant, apex, and flowers from 3-month-old plants; B, root, hypocotyl, cotyledon, and epicotyl of 19-d-old seedling; and C, pericarp obtained from 5-, 10-, 25-, and 35-d-old fruit, and from breaker (Br), turning (Tu), and ripe (RR) tomato fruits from wild-type and transgenic (3781^) plants. D, Hybridization of 1.9- and 2.1-kb fruit PME cDNAs (Turner et al., 1996) and *pmeu1* with the *pmeu1* and a fruit PME probes under the same hybridization conditions.

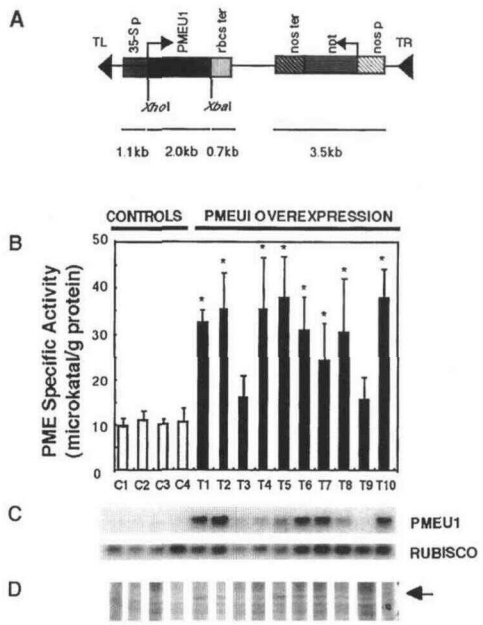

Figure 7. Expression of *pmeu1* under the control of the CaMV 35S promoter in transgenic tobacco plants. A, Schematic representation of the *pmeu1* chimeric gene construct pKYLXPMEU1. 35S.p, CaMV 35S promoter; PMEU1, *pmeu1* insert; rbcs.ter, Rubisco terminator; nos ter, nopaline synthase terminator; npt, neomycine phosphotransferase; nos p, nopaline synthase promoter; TL (TR), left (right) T-DNA borders. Arrows indicate the orientation of transcription. B, C, and D show the levels of PME specific activity, *pmeu1* RNA, and the 37-kD polypeptide (indicated by the arrow in D), respectively, in several independent transgenic tobacco plants transformed with vector pKYLX71 (C1–C4) or the chimeric *pmeu1* gene construct pKYLXPMEU1 (T1–T10). Total proteins and RNA were extracted from young tobacco leaves. PME specific activity is based on three independent extractions from each transgenic tobacco plant. The difference in PME specific activity between the controls and the transgenic plants at 99% confidence level (*t* test) is shown by asterisks. Northern analysis of total RNA was performed using a 0.8-kb DNA fragment from the 5′ end of *pmeu1* and a 800-bp cDNA corresponding to the small subunit of Rubisco. Fifteen micrograms of salt-extractable tobacco leaf protein was separated on SDS-PAGE and stained with Coomassie blue.

pKYLX71, probably representing the transcript of endogenous *pmeu1* homolog(s) in tobacco (data not shown). The difference in size between *pmeu1* mRNA in tomato and in transgenic tobacco plants is likely due to the presence of the *rbcS* terminator in the chimeric *pmeu1* gene construct.

Figure 7B shows that PME specific activity in transgenic tobacco plants expressing pKYLXPMEU1 was 1.5- to 4- fold higher compared with control plants containing pKLYX71. Although variable levels of *pmeu1* mRNA and PME specific activity in 10 independent transgenic tobacco plants were obtained, there was a correlation between the levels of *pmeu1* mRNA and increases in the PME specific activity. The wide range of increase in PME specific activity in transgenic tobacco plants is likely due to position effects observed in transgenic plants (Karpen, 1994). All transgenic plants showing accumulation of *pmeu1* mRNA and enhanced levels of PME specific activity contained an additional 37-kD polypeptide, as determined by SDS-PAGE analysis of protein extracts from young leaves (Fig. 7 D). The 37-kD polypeptide was not present in the tobacco plants transformed with pKYLX71 alone and did not cross-react with polyclonal antibodies directed against a fruit PME isozyme or neomycin phosphotransferase (data not shown).

Because of a blocked N terminus, we were not able to determine the N-terminal amino acid sequence of the 37-kD polypeptide. However, the 37-kD polypeptide partially purified from transgenic tobacco using differential salt extraction and heparin affinity chromatography demethoxylated citrus pectin (data not shown). These results indicate that *pmeu1* encodes a functional PME in transgenic tobacco plants. The accumulation of a 37- and not a 64-kD polypeptide suggests an extensive processing of a *pmeu1*-encoded polypeptide.

DISCUSSION

We have shown that *pmeu1*, a PME cDNA isolated from a tomato-leaf cDNA library (Gaffe et al., 1996), encodes an enzymatically functional PME. Transgenic tobacco plants expressing *pmeu1* under the control of the CaMV 35S promoter contained up to 4-fold higher PME specific activity compared with control tobacco plants, and the increase in PME specific activity in different transgenic tobacco plants was correlated with the accumulation of the *pmeu1* transcripts (Fig. 7). A heterologous system was selected to express *pmeu1* to avoid any problems due to homology-dependent gene silencing in transgenic plants (Meyer and Saedler, 1996).

To our knowledge, these results are the first evidence of the functional identification of a plant PME gene. A reduction in PME specific activity and an increase in the degree of methoxylation of pectins in transgenic fruits expressing a fruit-specific PME antisense gene have already provided indirect evidence of functional characterization of a fruit PME gene (Tieman et al., 1992; Hall et al., 1993). The identity of other plant PME genes is based on either similarities of their deduced amino acid sequence with the amino acid sequence of a tomato fruit PME (Markovic and Jornvall, 1986; Albani et al., 1991; Mu et al., 1994; Qiu and Erickson, 1995; Recourt et al., 1995; Richard et al., 1996) and partial amino acid sequence of a purified PME (Bordenave et al., 1996) or reactivity of the polypeptide encoded by a fruit PME cDNA to fruit PME antibodies (Harriman et al., 1991).

pmeu1 is the first example, to our knowledge, of a ubiquitously expressed PME gene. The PME homologs cloned from tomato fruit (Ray et al., 1988; Harriman et al., 1991; Hall et al., 1994), *B. napus*, petunia, and alfalfa pollens (Albani et al., 1991; Mu et al., 1994; Qiu and Erickson, 1995) show tissue-specific expression. ATPME1 from Arabidopsis is expressed in young but not in mature tissues (Richard et al., 1994). *pmeu1* transcripts are detected in all vegetative and reproductive tissues, with a higher level of accumulation in young (apex, expanding leaves, immature fruits, and flowers) than in older (expanded leaves and ripening fruits) tissues (Fig. 6).

The accumulation of *pmeu1* mRNA was not affected by fruit PME antisense gene expression in young fruits or in vegetative tissues, but a significant decrease was noted in older fruits (Fig. 6). We have ruled out the possibility that the observed *pmeu1* transcript accumulation in 35-d or older wild-type fruits was due to cross-hybridization with fruit-specific PME mRNAs (Fig. 6). A direct inhibition of *pmeu1* gene expression by the fruit-specific PME antisense gene is possible, especially since PMEU1 and fruit PMEs share a significant level of identity within certain regions (Fig. 2). However, since the fruit PME antisense gene is expressed in other tissues (Tieman et al., 1992), such an inhibition would be expected to occur in both fruit and vegetative organs. The decrease in *pmeu1* transcript level in developing transgenic fruits expressing the fruit PME antisense gene is correlated with the onset of fruit PME gene expression (Fig. 6).

Silencing of *pmeu1* in transgenic tomato plants by a constitutively expressed fruit PME antisense gene that differs significantly from *pmeu1*, only at the onset of expression of the fruit PME gene(s), could be of significance in understanding the mechanisms of gene silencing in transgenic plants. Antisense RNA-mediated gene silencing that is developmentally regulated or dependent on growth conditions has been reported in *Dictyostelium discoideum* and *Polysphodylium pallidum* (Sadiq et al., 1994; Funamoto and Ochiai, 1996). Although gene silencing by the heterologous antisense RNA genes has been shown, it seems to be less effective than homologous antisense RNA (Visser et al., 1991; Oliver et al., 1993).

None of the reported cases of antisense-mediated gene silencing has involved impaired expression of two isozyme genes differing significantly from each other by the antisense gene for one of the isozymes. Several mechanisms have been proposed to explain gene silencing by homologous and heterologous antisense RNA genes. These include degradation of mRNA and its antisense RNA duplex by double-stranded RNase, resulting in a reduction in the translatable pool of the target mRNA, inhibition of translation of the target mRNA due to formation of the double-stranded RNA duplex, impaired processing of the pre-mRNA, or involvement of cellular proteins (Oliver et al., 1993; Nellen and Sczakiel, 1996). Silencing of *pmeu1* in older fruits results in a failure of its transcript to accumulate and occurs only after the onset of fruit PME gene expression.

It is likely that a three-way sequential or parallel interaction among *pmeu1* mRNA, fruit PME mRNA, and fruit PME antisense RNA plays a role in the silencing of *pmeu1*. It is possible to envision mechanisms by which the action of a double-stranded RNase or other RNA-modifying enzymes on the duplex of fruit PME mRNA and its antisense RNA results in the production of a modified version of either fruit PME mRNA or fruit PME antisense RNA that destabilizes *pmeu1* mRNA or interferes with *pmeu1* mRNA processing, resulting in the silencing of *pmeu1*. In either mechanism this silencing would not be observed in vegetative tissues or in early fruit development due to undetectable expression of the fruit PME gene in these tissues (Fig. 6; Harriman et al., 1991). We are presently investigating some of these possibilities by characterizing levels of *pmeu1* mRNA and fruit PME mRNA and their antisense RNAs in the nuclei of transgenic fruits using reverse transcriptase-PCR. Additionally, we are characterizing expression of *pmeu1* and fruit PMEs in transgenic tomato plants expressing an antisense gene for *pmeu1* to understand the interaction between these two genes.

In vitro transcription-translation of *pmeu1* generated a polypeptide of about 64 kD (Fig. 1B). However, a 37-kD polypeptide that demethoxylated citrus pectin accumulated in all independent transgenic tobacco plants with increased levels of PME specific activity. Since the N terminus of the 37-kD polypeptide is blocked, more work is needed to demonstrate that this polypeptide is encoded by *pmeu1* in transgenic tobacco plants. Attempts are being made to deblock the 37-kD polypeptide to determine the N-terminal amino acid sequence. If these attempts are not successful, we will obtain the amino acid sequences of the internal polypeptides to establish the identity of the 37-kD polypeptide.

Molecular masses of PMEs purified from several plant species generally range between 22 and 42 kD (Sajjaanantakul and Pitifer, 1991), but PME isozymes with molecular masses of 54 and 55 kD have been reported in navel oranges and apples (Sajjaanantakul and Pitifer, 1991). We have purified two PME isoforms of 34 and 37 kD from tomato fruit and two PME isoforms of 37 and 42 kD from tomato roots (M.E. Tiznado, J. Gaffe, and A.K. Handa, unpublished results). However, many plant PME cDNAs reported to date, including tomato fruit PME cDNAs, encode polypeptides with calculated molecular masses ranging between 57 and 65 kD (Albani et al., 1991; Hall et al., 1994; Richard et al., 1994; Qiu and Erickson, 1995; Recourt et al., 1995; Gaffe et al., 1996; Glover et al., 1996; Turner et al., 1996). Taken together, these results suggest that polypeptides encoded by most plant PME genes undergo extensive processing to become the mature form of PME.

Our results show that the tomato genome contains at least two independent PME gene families: one for fruit PMEs, which has three copies in a tandem array (Turner et al., 1996), and a second one for *pmeu1*, which has one gene copy (Fig. 5). The PME gene expressed in pollen has not yet been described for tomato but has been cloned from *B. napus*, (Albani et al., 1991), petunia (Mu et al., 1994), and alfalfa (Qiu and Erickson, 1995). Low-stringency Southern analysis of tomato genomic DNA revealed the presence of up to 10 DNA fragments in the tomato genome that hybridized to a fruit PME probe (R.W. Harriman and A.K. Handa, unpublished data), suggesting that other tomato PME genes remain to be identified. Cross-hybridization of the *pmeu1* probe with potato and, to a lesser extent, with tobacco genomic DNAs and the high levels of similarity with Arabidopsis (Richard et al., 1996) and *V. radiata* (Bordenave et al., 1996) PME cDNAs suggest that *pmeu1* homologs are present in genomes of other Solanacae species and from such phylogenetically distant species as Arabidopsis and *V. radiata*.

Analyses of the deduced amino acid (Fig. 2) sequences revealed the presence of two domains in plant PMEs, a

variable N-terminal domain and a relatively conserved C-terminal domain. The variable N-terminal domains show only 12 to 19% identity, whereas the conserved C-terminal domains have 30 to 69% identity between different PMEs (Table I). PMEU1 and ATPME2 are an exception, sharing significant identity in both domains. The C-terminal domains of PMEs, including a PME gene from *E. chrysanthemi*, are enriched in β-sheets and contain several highly conserved amino acid motifs (Fig. 2). Similar enrichment in β-sheets has been noted in pectate lyases C and E and has been proposed to form a groove-like structure at the catalytic site of pectate lyase (Yoder and Jurnak, 1994).

The functional significance of the variable N-terminal domain in plant PMEs is not clear; however, based on the amino acid sequence of purified fruit PMEs, this region is cleaved off to produce the mature fruit PME proteins (Markovic and Jornvall 1986; Pressey and Woods, 1992). The N-terminal halves of most plant PMEs, including PMEU1 (Gaffe et al., 1996), contain several glycosylation sites. It is possible that these glycosylation sites are involved in the targeting of PME protein. The presence of a *N*-glycan signal that allows the polarized secretion of glycoprotein but is removed during the targeting process has been reported in eukaryotes (Hebert et al., 1995).

Preferential accumulation of *pmeu1* transcript in young, expanding tissues might correlate with changes in pectin chemistry recorded during plant cell elongation. Pectin esterification is maximal during the cell expansion phase and decreases as cell elongation ceases in carrot cell cultures (Knox et al., 1990; McCann and Roberts, 1994), soybean hypocotyls (Yamaoka and Chiba, 1983; Goldberg et al., 1986), and maize coleoptiles (Kim and Carpita, 1992). Also, immunolocalization of pectic compounds using monoclonal antibodies raised against low- and high-methoxyl pectic compounds (Knox et al., 1990) or the calcium pectate complex (Liners and Van Cutsem, 1992) indicate in muro de-esterification of the pectic matrix (McCann and Roberts, 1994; Roy et al., 1994).

The enrichment of low-methoxyl pectins and calcium pectate complexes at the intercellular junctions (Knox et al., 1990; Roy et al., 1994) suggest a role of PME in cell-to-cell cohesion and regulation of intercellular space, as indicated by the loss of tissue integrity in transgenic tomato pericarp depleted in the fruit-specific PME isoforms (Tieman and Handa, 1994). Higher levels of PME expression in young, expanding tissues might act as a regulator of cell wall extension and growth (Nari et al., 1991) or may create an anionic network, immobilizing cationic cell wall peroxidases involved in cross-reticulation of cell wall components (Macadam et al., 1992). We have created transgenic tomato plants over- and underexpressing *pmeu1* (J. Gaffe, M.E. Tiznado, and A.K. Handa, unpublished results). We are presently characterizing the effects of modified PME activity on pectin chemistry and growth phenotype of the segregating progeny from these transgenic plants. These studies should provide significant information concerning the role of *pmeu1* and pectin chemistry in plant growth and development.

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