JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 3465−3472

A Pectinmethylesterase Gene Associated with a Heat-Stable Extract from Citrus

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A putative thermostable pectinmethylesterase (TSPME) protein of 36 kDa was isolated from heat-treated citrus finisher pulp. After purification and partial sequencing of the protein, a reverse genetic approach was used to obtain the complete genomic sequence of a new pectinmethylesterase (PME) gene, *CsPME4*, from *Citrus sinensis* (L.) Osb. cv. Valencia. The *CsPME4* gene contained two exons of 1203 and 690 bp interrupted by a single positionally conserved intron of 1230 bp. A full-length *CsPME4* cDNA clone amplified from Valencia orange juice vesicles shared 98% identity with the genomic clone. The encoded protein of the full-length *CsPME4* cDNA shared 66 and 39% amino acid identity with the full-length encoded proteins of the citrus PME, CsPME1, and CsPME3, respectively, whereas the predicted mature protein of CsPME4 shared 80 and 61% identity with the predicted mature proteins of CsPME1 and CsPME3, respectively. Southern analysis demonstrated that *CsPME4* was present in at least two copies in the Valencia orange genome. Northern analysis revealed that *CsPME4* mRNA was accumulated mainly in young and developing tissues of Valencia orange. Several approaches to express recombinant CsPME4 in different systems failed to obtain active protein. Further research will be necessary to successfully express the putative TSPME gene *CsPME4* for biochemical characterization.

KEYWORDS: Citrus; heat-stable protein; pectinmethylesterase

INTRODUCTION

Pectinmethylesterase (PME) is a ubiquitous enzyme that facilitates plant cell wall modification and subsequent breakdown. PME catalyzes the demethylation of pectin, generating blocks of deesterified pectin and releasing hydrogen ion and methanol. The action of PME is thought to change the localized pH in the cell wall, thereby affecting structural interactions between cell wall components and activities of cell wall hydrolases involved in cell wall turnover and loosening (*1*). PME also participates in more specialized processes such as cell wall changes during fruit ripening (*2*), pollen tube growth (*3*), abscission (*4*), plant response to pathogen attack (*5*), and host-cell receptor recognition for the tobacco mosaic virus (TMV) movement protein (*6*).

In higher plants, PME occurs in multiple isoforms. Some PME isoforms are found specifically in certain cell types, whereas others are present in a wide number of tissues (*7*, *8*). Multiple PME isoforms have been reported in citrus. Citrus fruit PME isoforms have been the subject of numerous studies because of the negative impact of PME activity on juice quality. In fresh juice, PME activity leads to cloud loss in single-strength orange juices and gelation in their concentrates (*9*). Citrus juices are pasteurized to inactivate PME and ensure both microbial and physicochemical stability. Despite this heat treatment, however, some PME activity persists. Thermostable PME (TSPME) activity has been found in all citrus juices studied and can average 8.2% of total PME activity (*10*).

Studies regarding the identification, characterization, and control of TSPME isoform(s) have been done either to establish the physicochemical and kinetic properties of the enzyme(s) or to find new methods of inactivation. In this sense, several approaches have been proposed such as low pH inactivation (*11*), specific proteic PME inhibitors (*12*), inhibition by polyphenols (*9*), high-pressure treatments (*13*), supercritical carbon dioxide (*14*), or manothermosonication (*15*). However, none of these alternative methods have been adopted by the citrus industry due to undesirable side effects, incomplete inactivation, or economics. One approach to control could be down-regulation of the gene encoding TSPME. To down-regulate citrus TSPME isoform(s), the gene(s) that encode(s) TSPME must be isolated and characterized.

PME isoforms are encoded by different genes within a PME multigene family (*16*, *17*). In citrus, two complete PME genes were identified and characterized (*16*). The two *CsPME* genes contain two exons interrupted by a single large intron. *CsPME1* and *CsPME3* encode proteins with predicted sizes of 63.5 and 56.3 kDa, respectively. Alignment of the citrus deduced PME proteins with other deduced PME proteins revealed that higher plant PMEs in general contain a conserved C-terminal region associated with the mature active protein and a more variable region spanning one-third to half the length of the deduced

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10.1021/jf020146c CCC: $22.00
Published on Web 05/11/2002

protein's N terminus. *CsPME1* was expressed in young and/or rapidly growing tissues. In contrast, expression of *CsPME3* was low in these tissues but could be induced in abscission zones and leaf blades with ethylene treatment. Transient expression of the *CsPME3* open reading frame (ORF) in tobacco demonstrated that the CsPME3 protein was cleaved during processing (*18*). A 36 kDa cleavage product present on immunoblots was consistent with the size of mature, active PME protein isolated from citrus juice vesicles (*16*, *18*). Amino acid alignments of isolated and sequenced active plant PME proteins indicated that active protein is encoded by the predicted translation product of the conserved C terminus.

In our previous work, a reverse-genetics approach was used to identify PME genes on the basis of the amino acid sequence of an isolated citrus juice vesicle PME protein (*16*). In the end, it was unclear whether *CsPME1* or *CsPME3* encoded a TSPME protein. To identify TSPME gene(s), citrus finisher pulp, derived from juice vesicle membranes and cell walls during commercial juice extraction, was used to extract PME and enrich for TSPME protein (*18*, *19*). In this work, we report on the isolation, characterization, and expression of a citrus PME gene, *CsPME4*, identified using the amino acid sequence obtained from isolated TSPME protein.

MATERIALS AND METHODS

PME Isolation, Thermal Treatment, and PME Assay. TSPME forms were isolated as described previously by Braddock et al. (*19*). Briefly, citrus finisher pulp (150 g) from *Citrus sinensis* (L.) Osb. cv. Valencia was homogenized at 4 °C in an equal volume of 0.1 M Tris-HCl/1 M NaCl at pH 8.0. Unless otherwise noted, all procedures were conducted at 4 °C. The homogenate was stirred for 1 h and then centrifuged at 10000*g* for 20 min. The supernatant was saturated with 75% ammonium sulfate and stirred overnight. Precipitated proteins were recovered after centrifugation at 10000*g* for 20 min and solubilized in 50 mL of 10 mM Na_2PO_4, pH 7.0, buffer and then centrifuged at 10000*g* for 10 min. The supernatant was membrane-dialyzed (3.5 kDa MWCO, SpectraPor No. 3, Fisher Scientific, Atlanta, GA) twice for 8 h each against 2 L of 10 mM Na_2PO_4, pH 7.0. The dialysate was centrifuged at 10000*g* for 10 min. For TSPME isolation, 25 mL aliquots of the dialyzed extracts were heated for 30 min at 75 °C in a water bath. After cooling in an ice bath, samples were centrifuged at 22000*g* for 10 min to remove any denatured protein before preparative isoelectric focusing (PIEF). A portion of the supernatant, equaling 50−60 mg of protein, was separated with PIEF using a Rotofor equipped with the standard cell and run with pH 8.0−10.5 ampholytes (Bio-Rad Laboratories, Hercules, CA) as described previously (*16*, *19*). Each of the 20 PIEF fractions was assayed for PME activity by titration (*20*), and the proteins were separated with 10% SDS-PAGE. Enzyme activity was compared with the protein profiles of each fraction to identify the TSPME protein band. Protein concentrations were estimated according to the method of Bradford (*21*) using bovine serum albumin as standard.

PME Protein Digestion and Sequencing. PIEF fractions containing PME activity were pooled and separated on 12% SDS-PAGE. After silver staining of the reference gel to determine TSPME position, the band in nonstained gels containing the putative TSPME protein (200−300 pmol) was removed, crushed, and made into a slurry with 0.1% SDS, 50 mM Tris-HCl, 0.1 mM EDTA, and 200 mM NH_4HCO_3, pH 8.8, and then proteolytically digested with trypsin and endoprotease Lys-C (Sigma-Aldrich, St. Louis, MO). After proteolytic digestion, TSPME protein fragments were then electrophoresed on an 8% Tris−glycine gel. Four peptide fragments were selected for protein sequencing and transferred to nitrocellulose membrane. Micropeptide sequencing was performed at the Protein Sequencing Core Facilities at the University of Florida, Gainesville, FL.

Nucleic Acid Isolation and cDNA Synthesis. Genomic DNA was isolated from young leaves (5−6 months of age) of field-grown Valencia orange (*Citrus sinensis* cv. Valencia) trees according to the procedure of Dellaporta et al. (*22*). Lambda DNA was purified using the Wizard Lambda Prep DNA purification system (Promega, Madison, WI). Plasmid DNA was purified using the Wizard Mini Prep purification system (Promega). Total RNA was extracted from various tissues of Valencia orange, including juice vesicles, flavedo (the pigmented portion of the fruit peel), whole flowers, flower buds, anthers, petals, stigma plus ovary, calyx, young developing vegetative shoots, 8-week-old young fruit, and mature leaves (*16*). Poly(A)+ mRNA was purified from total RNA using the MicroPoly(A) Pure purification kit (Ambion, Inc., Austin, TX). First-strand cDNA was synthesized from 5 μg of total RNA or 1 μg of poly(A)+ mRNA (*23*).

Citrus TSPME Cloning. Degenerate oligonucleotide primers based on the amino acid sequence from the TSPME fragment corresponding to EGSSRRYI and DITFQNT were synthesized with the sequences 5′-GARGGNWSNWSNMGNMGNTAYAT-3′ and 5′-GAYATHAC-NTTYCARAAYAC-3′. These primers were used to amplify a 239 bp fragment from first-strand cDNA synthesized from juice vesicle total RNA. The fragment was cloned and sequenced. Sequence information was used to design two gene-specific primers that were used in a 5′ rapid amplification of cDNA ends (RACE) strategy (*24*). The RACE procedure was performed using the 5′/3′ RACE kit (Boehringer Mannheim, Munich, Germany) as recommended by the manufacturer. The 5′-RACE first strand was synthesized using gene-specific primer 5′-CTACAATAATCACCGGTAGCAGG-3′ followed by ligation of a linker sequence and amplification using a linker primer and the gene-specific 5′-GGAGTACAGAGAAAA-3′ as a nested primer. Temperature-cycling conditions were as follows: 2 min at 95 °C, followed by 34 cycles of 30 s at 94 °C, 30 s at 42 °C, and 1.5 min at 72 °C. Final extension was carried out at 72 °C for 10 min. An amplified product of 951 bp was cloned and sequenced.

Genomic Library Screening. The 951 bp RACE fragment was biotinylated using the Phototope system (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. The resulting probe was used to screen a previously existing *C. sinensis* cv. Valencia genomic DNA library (*16*). Genomic library lifts were screened as previously described (*23*). Positive clones were plaque-purified and subjected to restriction analyses.

DNA Sequence Analysis. DNA sequencing was performed at the University of Florida's DNA Sequencing Core Facility. Sequence comparisons were performed with the GenBank BLAST program (*25*). DNA and protein sequences were analyzed using the Omiga 2.0 software package (Oxford Molecular Ltd., Oxford, U.K.). Endoplasmic reticulum lumen targeting sequence was predicted using the SignalIP program in the World Wide Web Prediction Server Center for Biological Sequence Analysis (*26*). The PME motif was identified using the Motif program at GenomeNet, Japan (www.motif.genome.ad.jp). The O-glycosylation sites were predicted using the NetOglyc WWW Server (http://www.cbs.dtu.dk/services/NetOGlyc/).

Phylogenetic Analysis. Eighty amino acid residues containing the PME signature motif from the most conserved C-terminal region of 21 different plant PMEs were used for the phylogenetic analysis. Alignments were made using the Clustal (*27*) algorithm under default parameters (ClustalW, version 1.8). A phylogenetic tree was generated using the neighbor-joining method (*28*) included in the ClustalW program. To verify tree robustness, we used a heuristic search with 1000 replicates. Bootstrap values were calculated. The TreeView program (1.6.6, Roderic D. M. Page, 2001) was used to draw the tree.

Southern and Northern Blot Hybridization. Ten micrograms of total DNA was digested with the endonucleases *Eco*RI and *Eco*RI plus *Bam*HI (Promega) as recommended by the manufacturer. DNA restriction fragments were separated by electrophoresis in 0.8% (w/v) agarose Tris-HCl/acetic acid/EDTA (TAE) gels and transferred onto a Hybond-N membrane (Amersham Biosciences Corp., Piscataway, NJ) by capillary action using 10× sodium chloride−SDS−citrate (SSC). For northern analysis, mRNA was isolated from total RNA [Poly(A) Pure, Ambion, Inc.] and the amount quantified by spectrophotometry as described (*23*). One microgram of poly(A)+ mRNA was electrophoresed on a 1% formaldehyde−agarose gel and transferred to a Hybond-N membrane by capillarity using 2× SSC as transfer buffer. Membranes were dried, cross-linked, prehybridized, and hybridized as described previously (*16*) using a biotinylated 941 bp *Kpn*I fragment derived from the more variable N terminus of the PME sequence. Blots

were developed with the Phototope chemiluminescent detection kit (New England Biolabs Inc.) according to the manufacturer's protocol and exposed to X-ray film (Fuji Medical System USA Inc., Stamford, CT). Northern and Southern blots were repeated at least three times.

RESULTS

Isolation of a Putative TSPME Protein and Polypeptide Microsequencing. After thermal treatment, PME activity was recovered from fractions corresponding to isoelectric points (p*I*s) from pH 8.0 to 11.3 as previously described (*16*, *19*). When enzyme activity was compared with protein profiles of each fraction, a putative TSPME protein with a molecular mass of 36 kDa was identified (*19*) and isolated for protein digestion followed by amino acid sequence analysis. After digestion with trypsin and endoproteinase Lys-C (Sigma-Aldrich), four polypeptides were selected for microsequencing. Seven, 12, 14, and 18 amino acid residues were sequenced from the N terminus of each polypeptide. Amino acid sequences of the peptides were as follows: 7, AGEYREN; 12, GFLARDITFQNT; 14, APEGSSRRYIIRIK; 18, AVAAAPEGSSRRYIIRIK (**Figure 1**). The N-terminal sequence of these four polypeptides aligned with sequences located within the more conserved C-terminal region of deduced proteins derived from citrus PME (*16*) and other plant PME nucleotide sequences. Three of the four sequenced polypeptides aligned at different positions within the deduced protein of the C terminus (7, 12, and 18), whereas two polypeptides were overlapping (14 and 18).

Identification of a Genomic and cDNA Sequence of *CsPME4*, a Putative TSPME Gene. Alignment of the amino acid sequence data with deduced protein sequences of cloned PME genes allowed us to design degenerate primers to specific TSPME regions. These degenerate primers amplified a 239 bp fragment from cDNA prepared from Valencia orange juice vesicles. Alignment of the sequence of the 239 bp fragment revealed high homology with other citrus PME genes previously described. Using first-strand juice vesicle cDNA, a 951 bp fragment was amplified using a 5′ rapid amplification of cDNA ends (RACE) strategy. Cloning and sequencing of the 951 bp fragment indicated that this fragment aligned with the more variable N termini of the other citrus PME genes, *CsPME1* and *CsPME3* (*16*), but shared low homology. This suggested that the 951 bp fragment represented a unique Valencia citrus PME gene.

The 951 bp RACE fragment was used to screen a lambda Valencia orange genomic DNA library. Twenty-three positive clones were plaque-purified and examined by restriction analysis. Of these, two clones were selected for subcloning, Southern blot analysis, and sequencing. Both clones were nearly identical in nucleotide sequence, and one clone containing an 8.7 kb *Eco*RI insert was selected for further analysis. The genomic clone contained a complete PME gene. The new PME gene, *CsPME4* (GenBank accession no. AY040711), contained two exons of 1203 and 690 bp interrupted by a single positionally conserved 1230 bp intron. The genomic clone contained a 756 bp flanking 5′ regulatory sequence that contained a putative TATA box 80 bp upstream from the ATG start codon. A diagram comparing the structure of *CsPME4* with other citrus PME genes is presented in **Figure 2**.

A cDNA clone was amplified from first-strand cDNA made from mRNA isolated from Valencia juice vesicles by using gene-specific primers targeting the 5′-most end of exon 1 and the 3′-most end of exon 2 of the *CsPME4* ORF. The nucleotide sequence of the cDNA clone (GenBank accession no. AY040710) encoded a polypeptide of 633 amino acid residues (**Figure 1**). The cDNA clone was 98% identical to the genomic clone ORF. Alignment of the nucleotide sequences of genomic and cDNA clones indicated the positions of the exon and intron splice site junctions. The deduced protein of *CsPME4* cDNA predicted a polypeptide with a molecular mass of 69.4 kDa and p*I* of 8.2. The N-terminal region of CsPME4 contained a putative signal peptide for cellular targeting to the endoplasmic reticulum lumen (*29*), with a predicted cleavage site between amino acid residues 21 and 22. Recalculation of the molecular mass after removal of the putative signal peptide sequence gave a predicted molecular mass of 67.2 kDa and a p*I* of 8.0. The mature active deduced TSPME protein, derived from the more conserved C terminus of the larger 67.2 kDa protein, was estimated to contain 339 amino acid residues with a predicted size of 37.0 kDa and a p*I* of 8.2. This mature active deduced protein was similar to the size and p*I* estimated for purified Valencia finisher pulp TSPME protein. Furthermore, the CsPME4 deduced amino acid sequence perfectly matched amino acid sequences from peptides 7, 12, 14, and 18. No potential N-glycosylation sites were found in the putative mature protein sequence, although two potential O-glycosylation sites were predicted (**Figure 1**). The full CsPME4 encoded protein shared 66 and 39% amino acid identity with CsPME1 and CsPME3, respectively. The more variable N terminus of CsPME4 shared 50 and 25% identity, whereas the C terminus shared 80 and 61% identity with CsPME1 and CsPME3, respectively.

Genetic Characterization of *CsPME4*. Genomic Southern blots suggested that the Valencia orange genome contained at least two genes closely related to *CsPME4*. Under high stringency conditions (68 °C with 0.1× SSC, 0.1% SDS) the 941 bp *Kpn*I N-terminal probe hybridized with a unique pair of bands (**Figure 3**) that differed in size from hybridizing fragments in *CsPME1* and *CsPME3* (*16*). Weak cross-hybridization was seen in dot blots containing the full-length cDNA sequence of *CsPME1* and probed with the 941 bp *Kpn*I N-terminal fragment, whereas no cross-hybridization was observed with *CsPME3* (data not shown).

Accumulation of *CsPME4* mRNA in various Valencia orange tissues was determined by probing RNA gel blots with the 941 bp *Kpn*I N-terminal probe. A strong 1.9 kb hybridization band was observed in lanes containing young shoot, flower bud, petal, stigma, or ovary mRNA (**Figure 4**). Less intense hybridization occurred in lanes containing juice vesicle, anther, or young fruit mRNA. No hybridization was observed in lanes containing calyx, flavedo, or mature leaf blade mRNA.

Phylogenetic Analysis. Alignment of deduced amino acid sequences derived from the more conserved C terminus from 21 PMEs from higher plants were performed using ClustalW (*27*), and a phylogenetic tree was generated (**Figure 5**). Four major groups could be distinguished within the tree. Group I included two citrus PMEs, CsPME1 and CsPME4, together with two *Arabidopsis* PME sequences (AtPME2 and AtPME3) and one sequence from tomato (pmeu1). These genes had a general pattern of expression in the plant. Group II contained three PME isoforms expressed in pollen isolated from petunia, *Medicago*, and *Brassica* (Ppe1, P65, and Bp19). Group III clustered one peach PME sequence (Ppe8b), one *Arabidopsis* isoform (AtPME1), and one *Phaseolus* (PvRNAPE). These genes were expressed in ripening fruit (Ppe8b), young developing tissues (AtPME1), and pods (PvRNAPE). Finally, group IV included two tobacco isoforms (NpPME1MR and NpPME4MR), two potato isoforms (StBPE1 and StBPE2), two tomato isoforms (Pme21 and pB16), one PME *Phaseolus* isoform (PvVPE2), and one *Vigna* isoform (VrPECMEST). Group IV contained

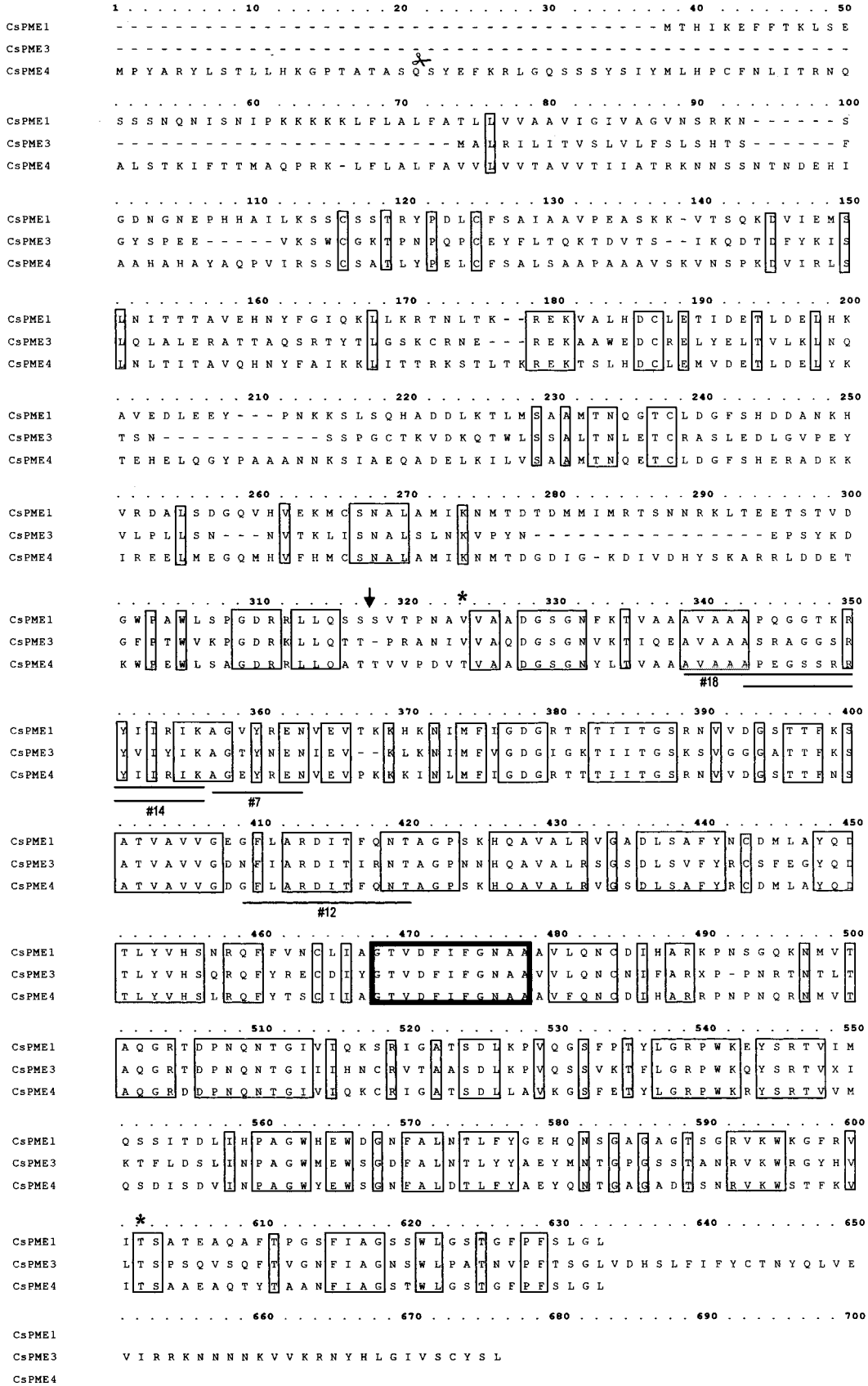

Figure 1. Alignment of deduced amino acid sequences of citrus PME: CsPME1, CsPME3, and CsPME4. Broken lines represent gaps introduced for best alignment. *CsPME4* amino acid residues corresponding to directly sequenced polypeptides 18, 14, 12, and 7 are underlined. The cleavage site of the putative N-terminal targeting sequence is indicated (scissors), as are amino acid sequence identities (boxes), predicted cleavage site for mature protein (arrow), CsPME4 O-glycosylation sites (asterisks), and PME motif (bold box).

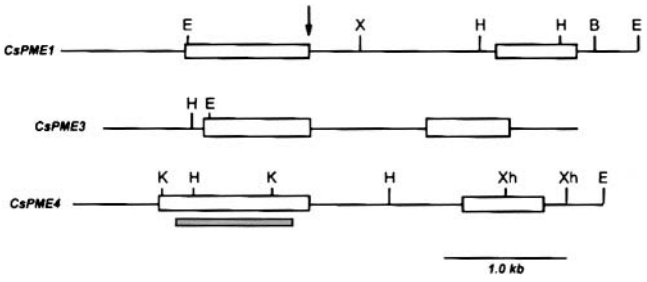

Figure 2. Schematic diagram of the cloned and sequenced *CsPME4* gene and its comparison with other citrus PME genes (*16*): ↓, positionally conserved exon–intron junction; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I; Xh, *Xho*I; gray bar, 951 bp RACE fragment and its alignment with the first *CsPME4* exon; open boxes, PME ORFs; horizontal lines, noncoding sequences.

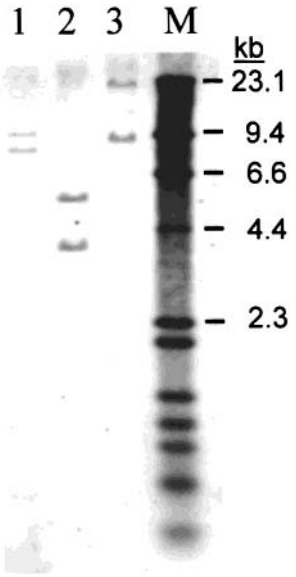

Figure 3. Southern hybridization analysis of Valencia orange genomic DNA. Ten micrograms of genomic DNA was digested with *Eco*RI (lane 1), *Eco*RI–*Bam*HI (lane 2) or *Bam*HI (lane 3) and hybridized with a 941 bp *Kpn*I N-terminal probe. M, molecular markers.

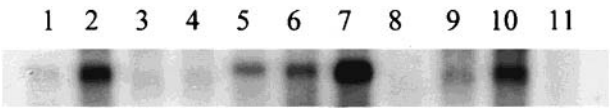

Figure 4. Northern hybridization analysis of *CsPME4* expression in various Valencia orange tissues (each lane contains 1 μg of mRNA); lane 1, juice vesicles; lane 2, stigma plus ovary; lane 3, calyx; lane 4, anthers; lane 5, petals; lane 6, flower buds; lane 7, whole flowers; lane 8, flavedo; lane 9, young fruit; lane 10, young developing shoots; lane 11, mature leaf blades. Blot was probed with a 941 bp *Kpn*I fragment derived from the 5′ end of the *CsPME4* cDNA.

PME genes isolated from either mature fruit or young embryos. Clustering of the PME genes into four distinct groups was supported by high bootstrap values. CsPME3 (citrus) and CpSPE1 (papaya) could not be assigned to any group based on bootstrap values.

DISCUSSION

Several studies have demonstrated the existence of a multigene PME family in higher plants. In citrus, different protein isoforms have been extensively characterized due to the negative impact of PME activity, in general, and TSPME activity, in particular, on citrus juices. However, despite the characterization

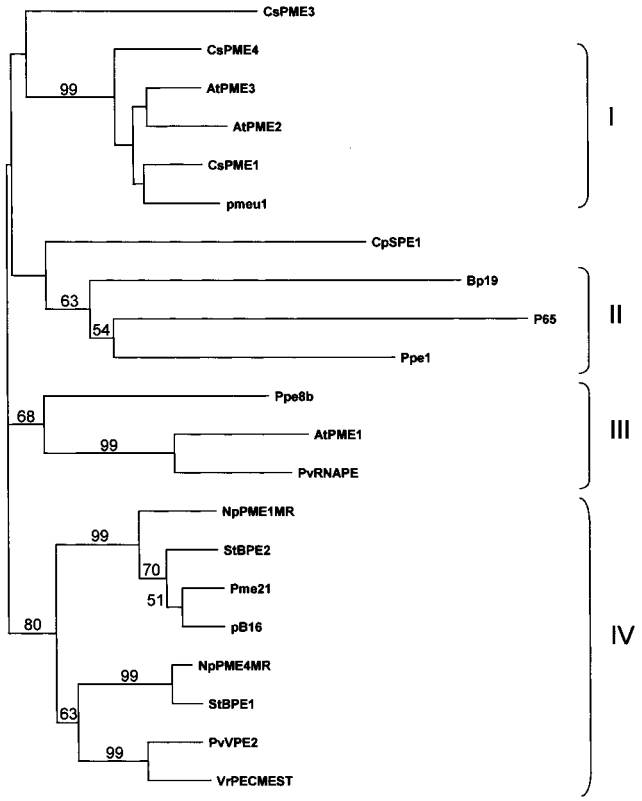

Figure 5. Phylogenetic analysis of different plant PMEs. The phylogenetic tree was generated on the basis of alignment of deduced amino acid sequences from the most conserved C-terminal region of 21 plant PMEs. Alignment was based on the Clustal algorithm (ClustalW; *27*), and tree was constructed on the basis of the neighbor-joining method (*28*). Values indicate the number of times (in percent) that each branch topology was found during the bootstrap analysis. Only bootstrap values >50 are shown. PME genes used to generate the phylogenetic tree and their GenBank accession numbers are as follows: CsPME3, U82977; CsPME4, AY040710; AtPME3, AF033204; AtPME2, U25649; CsPME1, U82976; pmeu1, U49330; CpSPE1, Y07899; Bp19, X56195; P65, U28148; PpE1, L27101; Ppe8b, X95991; AtPME1, X81585; PvRNAPE, X85216; NpPME1MR, Z71751; StBPE2, X97763; Pme21, U50986; pB16, X74639; NpPME4MR, Z71754; StBPE1, X97762; PvVPE2, X68029; VrPECMEST, X94443.

of several PME proteins from citrus, only three citrus PME genes have been isolated. Two genes, *CsPME1* and *CsPME3*, were isolated and characterized from Valencia fruit abscission zones and juice vesicles (*16*). Another citrus PME gene, isolated and characterized from citrus juice vesicles (*30*), shared 98.5% amino acid identity with *CsPME1* and is likely identical or very closely related. These studies did not attempt to identify a TSPME gene.

In previous work, we enriched for TSPME protein by heat-treating an extract from citrus finisher pulp (*19*). The 36 kDa TSPME protein associated with TSPME activity was isolated from the heated extract using PIEF and microsequenced. We used a reverse genetic approach to isolate a new PME gene, *CsPME4*, from Valencia orange finisher pulp that encoded this putative TSPME gene. The expected mature encoded protein molecular mass was 37 kDa with a p*I* of 8.2. The molecular mass of the putative TSPME agreed well with our empirical data; however, the p*I* was less than that previously reported (*19*). This shift in apparent p*I* could be due to association of additional proteins with PME during native PIEF, glycosylation, or other covalent associations with PME. Furthermore, the

cleavage site we predicted for the processed mature active protein was based on alignment with other PME sequences and therefore should be considered only an estimate of both molecular mass and p*I*. Polypeptide sequences directly obtained from the mature TSPME protein exactly matched deduced amino acid sequence from both the genomic and the corresponding cDNA clone.

Northern hybridization demonstrated that *CsPME4* was expressed in young developing stems, young fruit, stigma, ovary, flower petal, and floral bud tissues, suggesting that *CsPME4* activity may be important for growth and developmental processes in these tissues. In general, *CsPME4* expression was high and similar to expression demonstrated in young, developing tissues and floral tissues for *CsPME1* in citrus (*16*), *AtPME1* in *Arabidopsis* (*8*), and *PMEU1* in tomato (*31*). In contrast, the level of *CsPME4* expression was low in juice vesicle tissue. Juice vesicles are large, anatomically distinct sacs that contain hundreds of sugar- and acid-filled parenchyma cells surrounded by an epidermal layer (*32*). Christensen (*30*) immunolocalized a citrus PME protein with very high identity to *CsPME1* to the outer epidermal cell layers of juice vesicles. Thermostable PME activity in citrus juices represents ~8% of the total PME activity (*10*). If the TSPME gene were expressed in juice vesicles, its abundance would be expected to be proportionately low. However, the TSPME protein isolated in this study was obtained from rag, a heterogeneous plant material containing cell wall and membranous materials removed from the juice stream during the commercial juicing process. TSPME could be expressed in other fruit tissues such as seeds or citrus fruit segment membranes that contact juice upon extraction.

The *CsPME4* gene shared many structural similarities with *CsPME1*, *CsPME3*, and other higher plant PME genes. Alignment of both nucleotide and amino acid citrus CsPME sequences indicated that *CsPME4* was more similar to *CsPME1*. To estimate similarity and general functional relationships among PME genes, phylogenetic analysis was performed on 21 sequences encoding mature PME proteins from higher plants and citrus using the neighbor-joining method (*28*). As was previously reported (*17*, *31*), phylogenetic analysis indicated that tissue-specific and ubiquitously expressed PME genes have a high degree of similarity. *CsPME4* appeared to be closely related to a tightly clustered group of PME genes expressed in growing tissues, including *CsPME1*. In contrast, *CsPME3* did not share enough similarity to *CsPME1* and *CsPME4* to join that group and formed a separate branch. To our knowledge, *CsPME3* is the only PME gene strongly induced by ethylene (*16*). A second PME isoform that could not be assigned to any group was CpSPE1. Unfortunately, no information is available about CpSPE1 expression and, consequently, no conclusions can be inferred. Overall, our results reinforce the notion that the phylogenetic distribution of higher plant PMEs is based on functional rather than taxonomic relationships.

Overexpression of *CsPME4* using three heterologous expression systems was attempted to confirm thermostability of the CsPME4 protein product. Our first attempt was expression of *CsPME4* ORF in *Escherichia coli* using a low-expression vector (pBluescript II SK) to avoid formation of inactive enzyme due to insoluble inclusion bodies in the cells (*33*). After induction with isopropyl β-D-thiogalactopyranoside (IPTG), several *E. coli* proteins increased as indicated by SDS-PAGE. One of those corresponded to the expected mature protein size (37 kDa); however, no PME activity was detected in protein extracts.

A second attempt to express *CsPME4* was done using a *Pichia pastoris* heterologous protein expression system according to the method of Vandersall-Nairn et al. (*34*) (data not shown). Exogenous protein expressed in *Pichia* lacked any measurable activity, although several constructs with different translational start sites and yeast strains were used (*35*). Protein cleavage yielded a mature protein with the expected molecular mass and other smaller peptides, indicating that processing did not occur in a single step as was described previously in tobacco (*18*). Whether the loss of enzymatic activity was the result of protein misprocessing or other factors is uncertain. Finally, we used a TMV-based expression vector (*36*) containing the full *CsPME4* ORF to infect tobacco protoplasts and tobacco leaves. This transient expression system was previously used to obtain active CsPME3 from tobacco (*18*). However, the expression level of *CsPME4* was very low, and no differences in PME activity were observed between infected plants and controls (*35*). Expression of *CsPME1* was also attempted using the same TMV-based constructs but, again, no active protein was recovered. Why we were able to obtain active CsPME3 (*18*) and not CsPME1 or CsPME4 using the same expression system is not clear; however, *CsPME3* appears to be a unique plant PME with regard to sequence similarity and regulation. With the exception of *CsPME3*, to our knowledge there are no studies describing the expression of active PME using any nonplant or plant-transient expression system. The general lack of information regarding the overexpression of plant PME in simple model systems suggests that, during export to the apoplasm, PME isoenzymes may undergo organism-specific post-translational processing necessary for their structural and functional integrity (*37*).

In general, protein thermostability is thought to be a property acquired through subtle structural modifications in amino acid sequence, increased electrostatic forces such as hydrogen bonding, formation of ion pairs, and increased hydrophobic interactions that resist denaturization (*38*, *39*). Protein thermostability can also be affected by interaction with compounds in the surrounding milieu, such as heat shock proteins or other chaperones, carbohydrates, and polyols (*40*−*42*). In addition, a small but growing number of thermostable proteins belong to a group that contain little secondary structure, are conformationally flexible, and are functionally extended, the so-called "natively unfolded" proteins (*43*). Glycosylation has also been reported to increase the thermostability of some proteins (*44*). Cameron and Grohman (*45*) demonstrated the presence of N-glycosydic linkages in Valencia orange TSPME protein. However, putative N-glycosylation sites within the predicted mature protein of *CsPME4* are absent. This discrepancy suggests that more than one TSPME may exist in Valencia orange or that additional factors present in juice, such as chaperones or carbohydrates, may influence thermostability of native PMEs. Seymour et al. (*46*, *47*) established that grapefruit TSPME protein contained more carbohydrate than thermolabile PME and the moieties linked by O-glycosidic bonds. Two predicted O-glycosylation sites are located within the more conserved C terminus of the CsPME4-encoded protein. It is possible that O-glycosylation could play a role in CsPME4 thermostability. However, the role of glycosylation and structural changes brought about by subtle changes in amino acid sequence on the thermostability of *CsPME4* could not be assessed because of our inability to express CsPME4 in heterologous expression systems. Further research will be necessary to successfully express CsPME4 for biochemical characterization.

An important outcome of this work is the achievement of down-regulation of the putative TSPME gene, *CsPME4*, in Valencia orange. To minimize the potential of down-regulating

nontarget PME genes, a partial *CsPME4* fragment that encoded the polypeptide sequence within the more variable N terminus was used to build potential down-regulating constructs. Some of these constructs have been successfully introduced into callus lines of *C. sinensis* cv. Valencia. Protoplast transformation followed by somatic embryogenesis has allowed the regeneration of several transgenic plantlets. Like antisense PME tomato transformants (*2*), the growth of these young transgenic plants has so far not been affected by down-regulation with our *CsPME4* construct. Extensive analysis of regenerated plants will be conducted when their developmental stage allows full genetic characterization.

ABBREVIATIONS USED

PME, pectinmethylesterase; TSPME, thermostable pectinmethylesterase; BLAST, basic local alignment search tool; p*I*, isoelectric point; ORF, open reading frame; SSC, sodium chloride−SDS−citrate buffer.

ACKNOWLEDGMENT

We express our gratitude to Drs. Robert Braddock, Jude Grosser, Dennis Lewandowski, Oscar Olivares-Fuster, and Alison Vandersall-Nairn for their contribution to this work.

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Received for review February 4, 2002. Revised manuscript received April 17, 2002. Accepted April 17, 2002. This research was supported by the Florida Agricultural Experiment Station and grants from the U.S. Department of Agriculture (J.K.B., Award 00-35300-9391) and NATO Science Fellowship Program of Spain postdoctoral fellowship (C.R.A.) and approved for publication as Journal Series R-08451.

JF020146C