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Molecular Cloning of Tomato Pectin Methylesterase Gene and its Expression in Rutgers, Ripening Inhibitor, Nonripening, and Never Ripe Tomato Fruits[1]

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ABSTRACT

We have purified pectin methylesterase (PME; EC 3.1.11) from mature green (MG) tomato (*Lycopersicon esculentum* Mill. cv Rutgers) pericarp to an apparent homogeneity, raised antibodies to the purified protein, and isolated a PME cDNA clone from a λgtII expression library constructed from MG pericarp poly(A)$^+$ RNA. Based on DNA sequencing, the PME cDNA clone isolated in the present study is different from that cloned earlier from cv Ailsa Craig (J Ray *et al.* [1989] Eur J Biochem 174:119–124). PME antibodies and the cDNA clone are used to determine changes in PME gene expression in developing fruits from normally ripening cv Rutgers and ripening-impaired mutants ripening inhibitor (*rin*), nonripening (*nor*), and never ripe (*Nr*). In Rutgers, PME mRNA is first detected in 15-day-old fruit, reaches a steady-state maximum between 30-day-old fruit and MG stage, and declines thereafter. PME activity is first detectable at day 10 and gradually increases until the turning stage. The increase in PME activity parallels an increase in PME protein; however, the levels of PME protein continue to increase beyond the turning stage while PME activity begins to decline. Patterns of PME gene expression in *nor* and *Nr* fruits are similar to the normally ripening cv Rutgers. However, the *rin* mutation has a considerable effect on PME gene expression in tomato fruits. PME RNA is not detectable in *rin* fruits older than 45 days and PME activity and protein begin showing a decline at the same time. Even though PME activity levels comparable to 25-day-old fruit were found in root tissue of normal plants, PME protein and mRNA are not detected in vegetative tissues using PME antibodies and cDNA as probes. Our data suggest that PME expression in tomato pericarp is highly regulated during fruit development and that mRNA synthesis and stability, protein stability, and delayed protein synthesis influence the level of PME activity in developing fruits.

PME[3] (EC 3.1.11) is a cell wall-associated protein that demethoxylates pectin to form a carboxylated pectin while releasing methanol and a proton. PME activity and Ca^{2+} have long been implicated in causing the formation of a gel-like structure in the pectin of the middle lamella of plant cell walls. PME activity has been observed in all higher plants examined as well as in a number of plant pathogenic fungi and bacteria (5, 7, 15, 17, 28). Demethoxylation activity is associated with fruit ripening, cell wall maturation, abscission, and infection by plant pathogens (7, 19, 31). PME activity has been reported to increase during the development of avocado (1), apple (18), banana (4), and papaya (23) fruits. It is known that PME activity is present in immature tomato fruit and increases two- to threefold during ripening (12, 30, 34). Recently, Koch and Nevins (19) reported that the total uronic acid content of tomato fruit remains constant throughout ripening, whereas the degree of esterification decreases from 90% at mature green and breaker stages to 35% at the pink and ripe stages. Levels of PME activity in the ripening-impaired mutant Nr have been reported to be similar to those observed in normally ripening cultivars (13), whereas lower levels of PME activity have been observed in *rin* fruit (6, 14).

The exact role of PME in tomato fruit development and ripening is yet to be determined. However, it has been hypothesized that deesterification of pectin by PME and depolymerization by polygalacturonase are involved in fruit softening. This hypothesis is based on the observation that demethoxylation of pectin by PME causes a severalfold increase in cell wall solubilization by polygalacturonase (26, 32). To gain insight into PME and its role in tomato fruit development, we have purified PME from mature green fruits, raised antibodies to the purified enzyme, and isolated a PME cDNA clone. In this paper, we describe the characterization of PME expression during tomato fruit development at the enzyme activity, and protein and mRNA levels in normally ripening cv Rutgers and in the ripening mutants, *rin*, *nor*, and *Nr*. Results show that PME mRNA, protein, and activity are first detected between 10 to 20 d and continue to accumulate in the later stages of fruit development before showing a decline. Our data indicate that the *rin* mutation strongly affects expression of the PME gene during the later stages of fruit maturation, whereas the *nor* and *Nr* mutations have little effect on overall PME gene expression during fruit maturation.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill.) of the normally ripening cv Rutgers and ripening-impaired mutants *rin*,

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[3] Abbreviations: PME, pectin methylesterase; *rin*, ripening inhibitor; *nor*, nonripening; *Nr*, never ripe; PG, polygalacturonase; kb, kilobase(s).

nor, and *Nr* nearly isogenic to Rutgers (eight, four, and six backcrosses to Rutgers, respectively) were grown in the greenhouse and fruits harvested as described earlier (2). The inner contents of the fruit were excised, and the pericarp tissue frozen immediately by immersion in liquid N_2. Stem and leaf tissues were harvested from 5-week-old plants grown in greenhouse as described (2) and immediately frozen in liquid N_2. Root tissue was harvested from 5-week-old plants grown in sand and watered with a half-strength Hoagland solution. Roots were washed to remove sand and frozen in liquid N_2 immediately. All tissues were stored at −80°C until use.

PME Extraction and Activity Assay

Tissue samples were ground in liquid N_2, and proteins were extracted in an equal volume (w/v) of 2 M NaCl, pH 6, by stirring for 2 h at 4°C. The homogenate was centrifuged in a tabletop centrifuge to remove cell debris. Total protein present in the supernatants was determined by the method of Hartree (11) using BSA as a standard. The PME activity was determined using a Horizon 5997 automated pH titrator in a 25 mL reaction mixture (25°C) containing 0.5% citrus pectin (Sigma) in 150 mM NaCl, pH 7.5, and tissue extract. A 25 mM NaOH solution was used for titration. One unit of PME is the amount of enzyme that releases 1 μmol of carboxyl group min^{-1} under these conditions.

Purification of PME from Tomato Fruit

Mature green tomato pericarp was homogenized in an equal volume (w/v) of H_2O with a Waring blender at 4°C and centrifuged at 10,000*g* for 10 min. The pellet was resuspended in one volume of H_2O and homogenized with a Teckmar Tissuemizer, followed by centrifugation at 10,000*g* for 10 min. The pellet was extracted with one volume of 1 M NaCl at pH 6 for 2 h at 4°C and centrifuged at 10,000*g* for 10 min. The supernatant was fractionated by adding ammonium sulfate, and protein precipitated between 35 to 85% saturation was collected by centrifugation at 10,000*g* for 20 min at 4°C. The protein pellet was dissolved in ice-cold H_2O and dialyzed against 10 mM Mes, pH 6.5, and 150 mM NaCl overnight with two changes of the same buffer. The dialyzed protein sample was chromatographed on a CM-Sephadex C25 column (15 × 4 cm) and PME was eluted with a gradient of 0.15 to 1 M NaCl in 10 mM Mes, pH 6.5. Fractions were analyzed for PME activity and by SDS-PAGE. PME was first eluted at approximately 400 mM NaCl and continued to be eluted from the column as the salt concentration increased. Fractions with PME activity were pooled and concentrated by adding ammonium sulfate to 85% saturation (see Fig. 1B, lane 1). The pellet was dissolved in 5 mM sodium phosphate, pH 7.0, containing 50 mM NaCl and dialyzed overnight in the same buffer with a single change. CM-Sephadex-purified PME was diluted fivefold in 20 mM sodium acetate buffer, pH 4.3, and chromatographed over a SynChropak S300 HPLC column, a strong cation exchanger (SynChrom Inc., Linden, IN), using a Varian 5000 liquid chromatograph. Using a 0 to 500 mM NaCl gradient in 20 mM NaAc, pH 4.3, two peaks of activity were collected (Fig. 1A) and dialyzed against 5 mM sodium phosphate, pH 7.0, and 50 mM NaCl. SDS-polyacrylamide electrophoresis and Coomassie brilliant blue R-250 or silver staining were performed (3) to determine the purity of the PME sample (see Fig. 1B).

Preparation of PME Antibodies in Chickens

A chicken hen was immunized by injecting 50 μg of purified PME obtained from peak 2 of the HPLC S300 column (Fig. 1A) with Freund's complete adjuvant and boosted twice with Freund's incomplete adjuvant at 2-week intervals. Egg yolks from approximately 1 week prior to antigen injection and 2 weeks after the third antigen injection were used for preparation of immunoglobulin Y antibodies (33). Briefly, an equal volume of PS buffer (10 mM sodium phosphate, pH 7.5, and 0.1 M NaCl, containing 0.01% NaN_3) was added to the yolks and stirred. A 10.5% (w/v) solution of PEG-8000 (Sigma) in PS buffer was added to the yolk to yield a final concentration of 3.5% (w/v) PEG. The mixture was stirred for 30 min at room temperature and centrifuged at 12,000*g* for 20 min. The supernatant was filtered through two layers of cheesecloth into a graduated cylinder. A 42% (w/v) solution of PEG-8000 in PS buffer was added to make a final concentration of 12% (w/v) PEG. The mixture was stirred for 10 min and centrifuged at 12,000*g* for 20 min. The pellet was dissolved in the original yolk volume of PS buffer to which an equal volume of 4 M ammonium sulfate was added. The mixture was stirred for 30 min at 4°C and centrifuged for 20 min at 12,000*g*. The pellet was dissolved in P buffer (10 mM phosphate, pH 7.5, 0.01% NaN_3) and dialyzed overnight against the same buffer. Aliquots of the dialysate were stored at −80°C until used.

Quantification of PME Using Western Blot Analysis

Ten micrograms of total proteins extracted from root, stem, leaf, and pericarp tissues, as described above, were electrophoresed on 12% polyacrylamide gels containing SDS (2) and transferred to a nitrocellulose filter by semi-dry electroblotting. On the same gel, increasing amounts (0–500 ng) of purified PME protein were electrophoresed to obtain a standard curve to quantify the PME present in various samples. The blot was washed (22) for 15 min in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) and remaining sites were blocked with TBST containing 0.1% casein (TBST+c). The blot was incubated with PME-specific antibodies in TBST+c for 1 h at room temperature, washed three times for 10 min each in TBST+c, followed by incubation with alkaline phosphatase-conjugated immunoglobulin Y antibodies (Jackson Immuno Research Labs, Inc.) for 30 min. After washing three times in TBST+c, the blot was developed by an alkaline phosphatase-mediated reaction as described by Promega Biotech and scanned with a Beckman DU-8 spectrophotometer. The detection limit for immunoblotting was 10 ng of purified PME protein and a linear relationship was observed between 0 and 250 ng of purified PME protein and relative densitometric intensities. For each sample, relative densitometric intensities under each peak were determined and compared to a standard curve of purified PME to quantify the PME present in the sample.

Preparation of a λgtll cDNA Library from Mature Green Fruit Poly(A)$^+$ RNA and Isolation of a PME cDNA Clone

Poly(A)$^+$ RNA was extracted from mature green pericarp tissue according to Biggs and Handa (2). The cDNA library was constructed in λgtll as described (3, 16) and produced 3 × 10^6 recombinants from 6 μg mature green poly(A)$^+$ RNA starting material. The mature green cDNA library (30,000 recombinant plaques) was screened with anti-PME antibodies (16). The antibody binding and washing conditions were the same as used for Western blotting with the exception of removing antihost antibodies by incubating the antibody solution with lysed host extract containing nonrecombinant phage for at least 30 min at room temperature. The lysed host solution was generated by creating small-scale plate lysates with nonrecombinant λgtll as described by Maniatis *et al.* (20). Clones were purified through several screenings until all plaques produced a positive signal. A single, isolated plaque was then chosen for plate amplification (20). The putative cDNA clone was then multiplied by large-scale liquid amplification and purified by centrifugation through a 40% glycerol pad as described (20).

Characterization of a PME cDNA Clone Using DNA Sequencing

First attempts at isolating an *Eco*RI insert from the putative PME λgtll cDNA clone were unsuccessful due to the absence of an *Eco*RI site on the 5′ end of the cDNA clone. To circumvent this problem, the putative PME cDNA insert plus a fragment of the vector's β-galactosidase gene was excised with *Eco*RI and *Sac*I digestion and cloned into the *Eco*RI-*Sac*I sites in pTZ19U, a multipurpose plasmid produced by United States Biochemical. The *Eco*RI-*Sac*I fragment from this clone was purified on low melting agarose (FMC) and digested with *Sau*3A. The *Sau*3A-*Eco*RI fragment was cloned into *Bam*HI-*Eco*RI sites of pTZ19U using routine techniques (20). This clone was designated as PET1. DNA sequencing was performed using Sanger's dideoxy termination method (29) using the Sequenase Kit from United States Biochemical as described by the manufacturers. The DNA sequence was compared with the sequence of PME cDNA from tomato cultivar Ailsa Craig (27) using the Seqaid program.

PME mRNA Analysis

Total RNAs from root, stem, leaf, and pericarp tissues were extracted as described (2). Twenty-five micrograms of total RNA from each sample was denatured with formaldehyde, separated by electrophoresis on a 1.2% agarose gel under denaturing conditions, blotted to nitrocellulose, and analyzed as described (2). The PME cDNA insert from PET1 was ^{32}P-labeled with the random primers kit (BRL) and used as probe. The autoradiogram was scanned using a Beckman DU-8 spectrophotometer (2) to determine relative intensities of each band.

RESULTS

Protein Purification

PME and PG are both very abundant proteins in the ripe pericarp tissue of tomatoes, representing about 4.5 and 6%, respectively, of the proteins extracted in 1 M NaCl, pH 6 (3, this study). To avoid possible contamination with PG protein, we chose to isolate PME from mature green tomato pericarp that contains about 1.2% 1 M NaCl-extractable proteins as PME but no PG protein. Because PME remains bound to the cell wall under low salt, water-soluble proteins were removed by homogenizing the pericarp tissue in an equal volume of water before extracting with 1 M NaCl. NaCl-extractable proteins were fractionated using ammonium sulfate (between 35 and 85% saturation) and PME was further purified using a CM-Sephdex column. The CM-Sephadex-purified PME contained a contaminating protein of 37 kD in addition to several low mol wt proteins (Fig. 1B, lane 2). Repeated efforts to separate the 37 kD protein from 34 kD PME protein using gel filtration (P-150), DEAE-cellulose chromatography, and cationic native gel electrophoresis were unsuccessful (9). We were able to separate the PME from other proteins on HPLC using a SynChrom S300 column, a strong cation exchanger. Two major peaks of PME activity eluted from the S300 column (Fig. 1A). The smaller, first peak contained both the PME (34 kD) and 37 kD proteins (Fig. 1B, lane 4), whereas the prominent second peak contained only PME protein as determined by SDS-PAGE (Fig. 1B, lane 3). PME obtained from the second peak was used to raise PME antibodies in chickens (33).

Isolation of a Tomato PME cDNA Clone

The PME chicken antibodies were used to screen a cDNA library constructed in the expression vector λgtll using poly(A)$^+$ RNA from mature green tomato pericarp. Of 120,000 recombinants immunoscreened with PME antibodies, one recombinant produced a strong signal. This immunopositive plaque was purified and insert cDNA was subcloned as described in "Materials and Methods." The identity of the cloned insert cDNA was confirmed by DNA sequencing and comparing the determined sequence with a published PME cDNA sequence (Fig. 2A) (27). This PME cDNA clone, which contained 674 base pairs, was designated as PET1. The DNA sequence of the PME cDNA, PET1, is 90.6% similar to the published PME cDNA sequence (27). The deduced amino acid sequence of PET1 shows about 94% similarity with the rearranged primary amino acid sequence for the PME protein reported by Markovic and Jornell (21) (Fig. 2B, Table I).

Changes in PME Activity, Protein, and mRNA during Development of Tomato Fruit

PME activity was present in all stages of fruit development tested (Fig. 3A). The lowest level of PME activity was detected in 10-d-old fruit. The level of PME activity increased until a maximum was reached at the turning stage of fruit development (Fig. 3A). During early stages of fruit development, the PME activity parallels the increase in PME protein detected by immunoblotting except that PME protein was not detected in the pericarp of 10- and 15-d-old fruits (Fig. 3A, B). PME protein was first detected in 20-d-old fruit and levels of PME protein increased gradually until the ripe stage (Fig. 3). Between the mature green and breaker stages, there was an

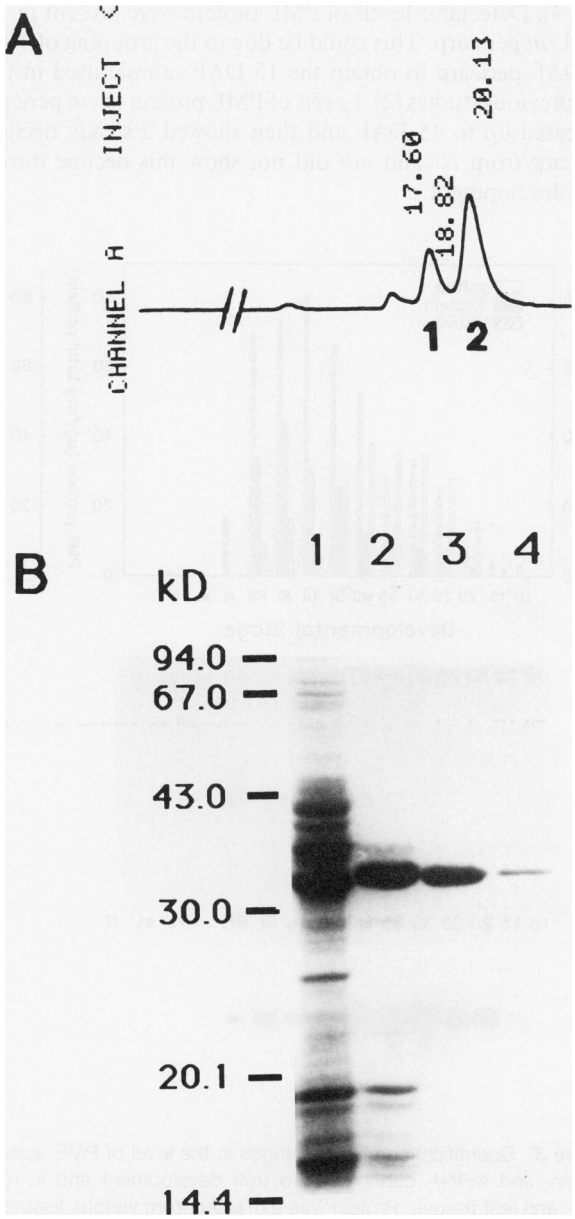

Figure 1. A, Elution profile of PME from SynChropak S300 strong cation exchange column. The HPLC SynChropak S300 column was equilibrated with 20 mM Na acetate, pH 4.3, and CM-Sephadex-purified PME was chromatographed as described in "Materials and Methods." Elution peaks 1 and 2 represent absorbance at 280 nm. Numbers indicate time (min) of elution for each peak from the Syn-Chropak S300 column. B, SDS-PAGE analysis of various fractions during PME purification. Gel was stained with Coomassie brilliant blue. Lanes: (1) 35 to 85% ammonium sulfate fraction; (2) CM-Sephadex C25-purified PME; (3) peak 2 from HPLC S300; (4) peak 1 from HPLC S300.

approximately twofold increase in PME protein (Fig. 3A, B). The highest levels of PME protein were present in the pericarp of ripe fruit, and based on a standard curve for purified PME represented about 4.5% of the total protein extractable in 1 M NaCl, pH 6, from the pericarp of the ripe fruit.

An RNA species of about 1.95 kb present in the total RNA from tomato pericarp hybridized to the radiolabeled insert from PET1 and was absent in root, stem, and leaf (Fig. 3C). PME mRNA was first detected in 15-d-old fruit and reached a maximum steady-state level in fruit that was 30 d old (Fig. 3A, C). PME mRNA levels remained elevated through the mature green stage (mature green fruit is approximately 40 d

A

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PET1     GGAAGCTGATGGAGAGTTCGGGTAAGGACATTATAGCGAATGCAGTGGTGGCAAAAGATG
ENG-PME ------------------------------------GG--------------------------

PET1     GAACAGGGAATTATCAAACACTTGCTGAAGCAGTTGCTGCAGCACCAGATAAGAGTAAGA
ENG-PME ----------A----G---------------T----------------------------

PET1     CGCGTTATGTAATTTATGTAAAGAGGGGAACTTATAAAGAGAATGTTGAGGTGGCTAGCA
ENG-PME ------------------------------------------------------------AG-----

PET1     ATAAAATGAACTTGATGATTGTTGGTGATGGAATGTATGCTACGACCATTACTGGTAGCC
ENG-PME GG--------T---------A----------C-----------C-T---------G----

PET1     TTAATGTTGTCGAAGGATCAACAACCTTCCGCTCTGCCACTCTTGCTGCAGTCGGCCAAG
ENG-PME ------------T----------------A----------------T---A---

PET1     GATTTATACTACAGGACATATGTATACAGAACACAGCAGGGCCAGCGAAAGACCAAGCAG
ENG-PME ------------------------------------------A-----T---C-------T-

PET1     TGGCACTTCGAGTTGGAGCTGATATGTCTGTCATAAATCGTTGTCGTATCGATGCTTATC
ENG-PME -T----------------------A-----------------------------------

PET1     AAGACACCCTTTATGCACATTCTCAAAGGCAATTCTATCGAGACTCCTACGTGACAGGGA
ENG-PME ----------------------------------------AGAG----------------

PET1     CTGTTGATTTCATATTTGGTAATGCAGCAGTTGTATTCCAGAAATGCCAGCTCGTAGCTA
ENG-PME --A----------C-------------------------------------------------

PET1     GAAAACCGGGTAAATACCAGCAAAACATGGTGATCGCACAAGGCAGGACGGACCCAAATC
ENG-PME ---------------------------------CT--------------------------

PET1     AGGCCACGGGGACATCAATTCAGTTCTGTAACATAATAGCAAGTTCGGACCTAGAACCAG
ENG-PME -------------------------T---G-T----------C-T------A------

PET1     TCCTGAACGAATTC
ENG-PME --G----A------
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B

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PET1     KLMESSGKDIIANAVVAKDGTGNYRTLAEAVAAAPDKSKTRYVIYVKRGTYKENVEVASN
M-PME            --------Q----D-Q-----------------------------------
ENG-PME ----------G----------K---------------------------------S-R

PET1     KMNLMIVGDGMYATTITGSLNVVEGSTTFRSATLAAVGQGFILQDICIQNTAGPAKDQAV
M-PME    -...-------------------------------------TL-------------R----
ENG-PME ------I-------I--------D-----H--------K----------------H---

PET1     ALRVGADMSVINDCRIDAYQDTLYAHSQRQFYRDSYVTGTVDFIFGNAAVVFQKCQLVAR
M-PME    ------------R---------------------D----------G----KN-------
ENG-PME -------K----R-------------------QS------I-------------------

PET1     KPGKYQQNMVIAQGRTDPNQATGTSIQFCNIIASSDLE.PVLNEF
M-PME    ----------T----------------------G---R--..--
ENG-PME ----------T-----------------D----P--K.--VK--
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Figure 2. A, DNA sequence of a partial PME cDNA clone (PET1) and its comparison with a PME cDNA clone (ENG-PME, nucleotide 316 to 989) isolated by Ray *et al.* (27). Dashes represent homologous nucleotides and mismatched nucleotides are shown. B, Comparison of deduced amino acid sequences from PET1 with the rearranged primary amino acid sequence of tomato PME determined by Markovic and Jornell (21) (M-PME) and deduced amino acid sequence from ENG-PME (nucleotide 318 to 989) isolated by Ray *et al.* (27). The amino acid sequence of M-PME begins with the published mature N-terminal of tomato PME (21). The continuity of the amino acids in the published PME polypeptide chain (21) was rearranged to align the primary amino acid sequence to the deduced amino acid sequences of PET1 and ENG-PME (27). See text for details. Mismatched amino acids are shown. Dashes represent homologous amino acids and dots represent absence of amino acids.

Table I. *Comparison of PME Amino Acid Similarity Deduced from Two PME cDNA Clones and a Determined PME Primary Amino Acid Sequence*

PET1 is the PME cDNA isolated in the present study; ENG-PME represents the PME cDNA isolated by Ray *et al.* (27); and M-PME is the rearranged (Fig. 2) primary amino acid sequence of tomato PME reported by Markovic and Jornell (21).

Amino Acid Sequences	Conserved	Total	Mismatched	% Identity
ENG-PME/PET1	203	224	21	90.6
ENG-PME/M-PME	184	210	26	87.6
PET1/M-PME	197	210	13	93.8

old) and declined as the fruit ripened (Fig. 3A, C). Interestingly, the levels of PME mRNA declined just before a twofold increase in PME activity and protein during the ripening phase.

Levels of PME Activity, Protein, and mRNA in Tomato Root, Stem, and Leaf Tissues

Stem and leaf tissue contained PME activity similar to 10- and 15-d-old fruit, whereas root showed PME activity levels comparable to 25-d-old fruit (Fig. 3A). However, no detectable levels of PME protein and mRNA were found in root, stem, or leaf tissues using antibodies raised against fruit PME and the PME cDNA cloned using fruit mRNA as probes, respectively (Fig. 3B, C). These results suggest that different isozyme(s) of PME are present in these tissues that are not recognized by fruit-specific PME antibodies and cDNA probes.

PME Gene Expression during Mutant Pericarp Development

Pericarp from homozygous *rin, nor,* and *Nr* ripening mutants were similarly examined for PME gene expression during fruit development (Fig. 4). However, fruits from these tomato mutants do not undergo normal ripening. This makes it difficult to compare between the expression of PME in the late stages of mutant fruit development and the various stages of wild-type fruit ripening. Thus, in the present investigation we have characterized the expression of PME in mutant fruits based on fruit age (up to 75 DAF). In general, fruits from Rutgers reach a fully ripe stage by 50 ± 5 DAF.

PME activity profiles during the early stages of fruit development (up to 45 DAF) in *rin, nor,* and *Nr* pericarp were similar to that observed in the normally ripening cultivar Rutgers, but were different in the later stages of fruit development (Fig. 4). Pericarp from *rin* showed a sharp decline in PME activity in the later stages of fruit development (after 45 DAF). The profile of PME activity in *nor* pericarp was similar to the Rutgers pericarp, with a significant increase in PME activity between the 55 and 65 DAF stages. Developing pericarp from *Nr* fruits showed a pattern similar to *nor* fruits but contained lower levels of PME activity with compared to *nor* fruits. As compared with Rutgers fruits, which show maximum PME activity at the turning stage of fruit ripening (which occurs about 45 DAF), the maximum measured PME activities in *Nr* and *nor* fruits were observed between 65 and 75 DAF (Fig. 4).

Changes in PME protein reflected observed changes in PME activity during the development of pericarp in mutant fruits (Fig. 4). Detectable levels of PME protein were present in 15-d-old *rin* pericarp. This could be due to the grouping of 10 to 20 DAF pericarp to obtain the 15 DAF sample used in this and previous studies (2). Levels of PME protein in *rin* pericarp increased up to 45 DAF and then showed a steady decline. Pericarp from *Nr* and *nor* did not show this decline during fruit development.

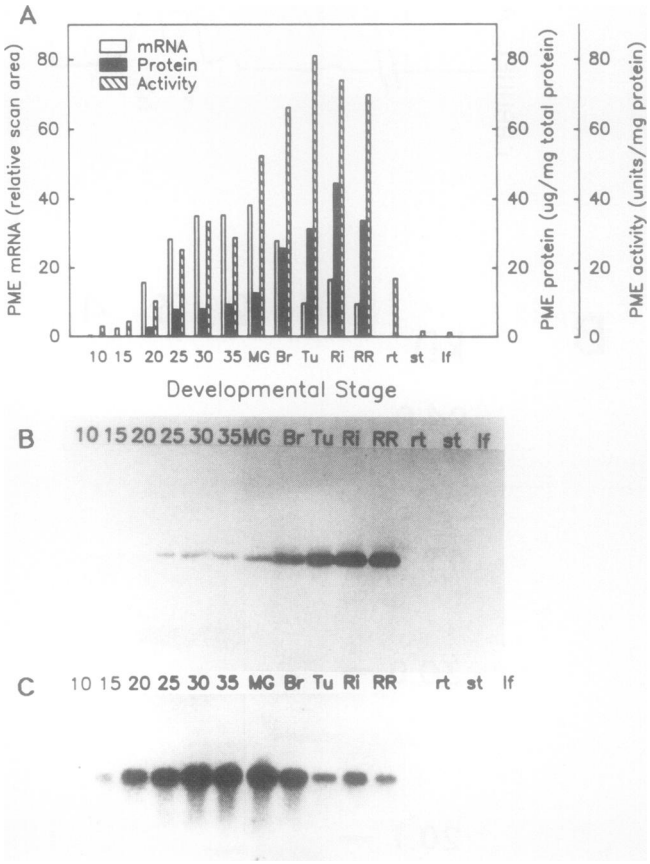

Figure 3. Quantification of the changes in the level of PME activity, protein, and mRNA during tomato fruit development and in root, stem, and leaf tissues. Protein was extracted from various tissues of normally ripening Rutgers in 1 M NaCl, pH 6, and assayed for PME activity using an automated pH titrator. The immunoblot, shown in B, was scanned with a Beckman DU-8 spectrophotometer to quantify PME protein in samples. For immunoblots, 10 μg of 1 M NaCl-extractable protein for each sample was electrophoresed on 12% SDS-PAGE gel and PME protein was detected using PME antibodies as described in "Materials and Methods." Purified PME protein (0–500 ng) was used to create a standard curve. The Northern blot autoradiogram, shown in C, was scanned with a Beckman DU-8 spectrophotometer and the relative intensities were determined (arbitrary units). Twenty-five micrograms of total RNA from each sample was used for Northern blot analyses. Numbered samples indicate age (DAF) of tomato fruits, and ripening stages are depicted by abbreviations: MG, mature green; Br, breaker; Tu, turning; Ri, ripe; RR, overripe. rt, st, and lf represent root, shoot, and leaf tissue, respectively.

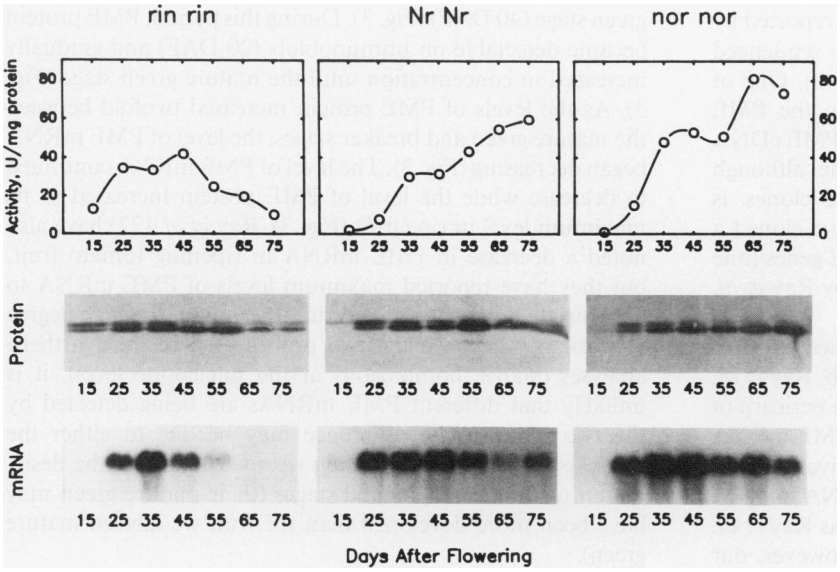

Figure 4. Changes in PME activity, protein, and mRNA in developing tomato fruit of the ripening mutants *rin*, *nor*, and *Nr*. Flowers were tagged at pollination and fruits were harvested at various DAF. Fruits harvested throughout 10 d intervals of age were grouped and midrange age was used to indicate age. For example, the 25 DAF sample represents pericarp from fruits harvested between 20 and 29 DAF. Other details are the same as in Figure 3. Numbers indicate age (DAF) of fruits.

PME mRNA levels in *rin* pericarp increased during the first 35 d of fruit development, a pattern similar to that observed in Rutgers pericarp. However, thereafter the PME mRNA levels decreased rapidly and were barely detectable in 55, 65, and 75 DAF pericarp. This pattern of PME mRNA in developing *rin* pericarp was different from that of PME activity and protein (Fig. 4). Although both PME activity and protein showed decreases during the later stages of *rin* pericarp development, they were present until 75 DAF. This result suggests that PME protein has a relatively long half-life in *rin* tomato fruits. However, other more elaborate mechanisms, such as translational efficiency of mRNA during fruit development, should also be considered. Pericarp from *Nr* and *nor* genotypes exhibited changes in PME mRNA levels similar to those observed in pericarp from normally ripening Rutgers (Figs. 3C, 4). The PME mRNA levels remained elevated until 45 DAF and then showed a steady decline as fruits from *Nr* and *nor* matured.

DISCUSSION

PME activity in ripening tomato fruit has been studied by a number of research groups (6, 12, 25, 30, 34). The presence of multiple forms of PME in tomato fruit has been noted by several investigators. Pressey and Avants (24) examined pericarp protein extracts from four different cultivars of tomatoes (Green Marion, Homestead, Ripe Marion, and Pixie) by passing the protein samples through a DEAE-Sephadex A50 column and assaying for PME activity. Four peaks of PME activity were resolved and labeled PMEI through PMEIV based on elution profiles. PMEIV was the most abundant form in all cultivars with the exception of Pixie. Tucker *et al.* (34) also used DEAE-Sephadex A50 separation to study "isozymes" of PME from the cultivar Ailsa Craig and reported two forms of PME (PME1 and PME2) in both mature green and ripe fruits. They showed that PME1 decreases slightly as the fruit ripens, whereas PME2 increases roughly twofold from the mature green to the ripe stage (34). Delincee was able to detect up to eight molecular forms of tomato PME when thin layer isoelectric focusing was combined with activity staining (8). By passing the CM-Sephadex-purified PME through the HPLC S300 column, we are able to obtain two peaks of PME activity. The first peak comprises two proteins (the 34 kD PME and 37 kD proteins), and the second peak contains only the 34 kD PME protein (Fig. 1). At present, we do not know if the PME present in the first and second peaks are different isozymes or the result of a molecular association between PME and the 37 kD protein that influences the elution from the S300 column, giving rise to two peaks of activity. Because of the use of different ion exchange columns (DEAE-Sephedex A50 by Tucker *et al.* [34] and HPLC S300 in the present study), it is not possible to say that the PME1 and PME2 proteins separated by the HPLC S300 column are the same as the PME1 and PME2 proteins reported by Tucker *et al.* (34). A biochemical explanation for the presence of multiforms of PME has not yet been established. However, based on analysis of genomic DNA clones, we have shown the presence of at least three PME genes in the tomato genome (9, 10).

The deduced amino acid sequence from PET1 could be aligned to the published primary amino acid sequence of tomato PME (21) only after rearranging the peptide fragments of the PME polypeptide chain, a result similar to that observed by Ray *et al.* (27). As discussed by Ray *et al.* (27), this discrepency is likely due to misinterpretation of peptide overlap in the direct primary amino acid sequence analysis rather than posttranslational rearrangement of the PME precursor polypeptide. The deduced amino acid sequence of PET1 is most similar (93.8%) to the rearranged PME amino acid sequence reported by Markovic and Jornell (21) (M-PME in Fig. 2 and Table I) and is somewhat less similar (90.6%) to the deduced amino acid sequence of PME reported by Ray *et al.* (27) (ENG-PME in Fig. 2 and Table I). However, the PET1 sequence shows greater similarity to both M-PME and ENG-PME than M-PME and ENG-PME show to each other (Table I). The comparison of our PME cDNA and the PME cDNA isolated by Ray *et al.* (27) with the published PME protein sequence of Markovic and Jornell (21) indicates that

we have cloned a different PME cDNA from that reported by Ray *et al.* (27). We now have cloned and partially sequenced three genomic clones of PME from tomato (9, 10). One of these genomic clones appears to be identical to the PME cDNA cloned in the present study, another to the PME cDNA cloned by Ray *et al.* (27). The third genomic clone, although showing strong similarity to the other two PME clones, is different (9, 10). These data indicate that we have cloned a different PME cDNA clone and at least two PME genes (one cloned in the present study and another cloned by Ray *et al.* [27]) are expressed in developing tomatoes.

On denaturing formaldehyde gels, the pericarp PME mRNA migrated as a single band of about 1.95 kb. Ray *et al.* have reported the PME mRNA isolated from ripe pericarp of Ailsa Craig to be 1.6 kb (27). This difference in PME mRNA size may be attributed to the difference in cultivars or the choice of mol wt standards. We used BRL RNA mol wt markers as standards in the present study, whereas Ray *et al.* (27) employed denatured DNA as standards. However, our attempts to use denatured DNA as mol wt markers for RNA sizing have yielded estimates that were smaller than those obtained with RNA standards.

PME activity is observed much before the tomato fruit begins to visibly ripen. Using tagged flowers, it was possible to obtain fruits of known ages and to determine that PME protein and activity begin increasing approximately 20 DAF. The PME activity continues to increase until the turning stage and declines thereafter (Fig. 3A). Maximum levels of PME activity in either the breaker or turning stage of fruit ripening have also been observed by Hobson (12), Pressey and Avants (25), and Sawamura *et al.* (30). Although maximum PME activity was found in turning stage fruit, maximum levels of PME protein did not occur until the ripe stage (Fig. 3A). Western blots of protein samples from later stages of fruit ripening show an apparently intact protein (Fig. 3B) and do not appear to represent a decrease in specific activity resulting from proteolysis. Because pectin is only 35% methylated in the ripe fruit (19), it would be interesting to measure PME activity with pectins of defined esterification levels. It is possible that PME present in later stages of ripening is more active against pectin with a lower degree of esterification.

Stem and leaf tissue exhibited PME activity levels comparable to 10- and 15-d-old fruit, whereas root tissue contained elevated levels of PME activity. However, it was not possible to detect PME protein in any of these tissues on immunoblots with our PME antibodies, even using four times the level of detectable PME from fruit tissue. The inability to detect PME protein with anti-PME antibodies, even though PME protein was observed in fruit containing lower levels of PME activity, suggests that there may be tissue-specific isozymes of PME. This is supported by the lack of hybridizable PME mRNA in root, stem, and leaf tissues (Fig. 3C). However, more work is needed, especially by increasing sensitivities of both immuno- and RNA-blots, to confirm the absence of fruit-specific PME expression in other parts of the tomato plant.

PME expression at the mRNA level provided some interesting results that do not strictly correlate to PME protein and activity levels. PME mRNA was first detected in 15-d-old fruit, increased to a maximum level in 30-d-old fruit, and remained at its elevated steady-state level until the mature green stage (40 DAF) (Fig. 3). During this period, PME protein became detectable on immunoblots (20 DAF) and gradually increased in concentration until the mature green stage (Fig. 3). As the levels of PME protein increased twofold between the mature green and breaker stages, the level of PME mRNA began decreasing (Fig. 3). The level of PME mRNA continued to decrease while the level of PME protein increased to its maximum level in ripe fruit (Fig. 3). Ray *et al.* (27) have also noted a decrease in PME mRNA in ripening tomato fruit, but they have reported maximum levels of PME mRNA to occur in the immature green fruit. Because of the high degree of similarity between the two probes used in the Northern analyses (90.6% homologous at the nucleotide level), it is unlikely that different PME mRNAs are being detected by the two groups. The difference may be due to either the cultivars employed (Ailsa Craig *versus* Rutgers) or the designation of the developmental stages (their mature green may have been more developed than the fruit we labeled mature green).

The expression studies of PME activity, protein, and mRNA point to several levels of gene control. The inability to detect PME mRNA using a tomato fruit PME cDNA as a probe in root, stem, and leaf tissues suggests transcriptional tissue-specific regulation of the PME gene. Steady-state levels of PME mRNA may also be influenced by RNA stability. Nuclear run-on experiments should be conducted to determine if *de novo* synthesis of PME mRNA is occuring and if changes in the rate of transcription during fruit development can account for the elevated levels of PME mRNA found in 30- to 40-d-old fruit. Because of the sudden accumulation of PME protein between the mature green and breaker stages, translational control also appears to play a role in PME gene expression. The unexplained decrease in PME's specific activity in later stages of fruit ripening may indicate another level of control of PME activity.

PME gene expression during development of pericarp from mutants *rin, nor,* and *Nr* indicate that these mutations, especially *rin,* affect expression of the PME gene during the development of tomato fruits. Effects of the *rin* mutation are apparent by 35 DAF, whereas the overall levels of PME activity, protein, and mRNA in *Nr* pericarp are lower than *nor* pericarp during fruit development. It has been reported that different isozymes of PME are expressed during tomato fruit development (34). Analysis of PME genomic clones indicates the presence of at least three PME genes in tomato (9, 10). Based on DNA sequence analysis of cloned PME cDNAs, at least two of these genes are expressed during fruit development. Is it possible that only one of these genes is expressed in *rin* fruits? This would explain the lower levels of PME gene expression in *rin* pericarp. Using oligonucleotide probes specific to the three PME genes that we have cloned and sequenced, we are currently studying the expression of different PME genes in the development of mutant fruits. Based on PME expression in *rin* pericarp, our studies also indicate that PME protein has a relatively long half-life. Even though no PME mRNA was present in 55-d-old fruits, PME protein and activity were observed in 75-d-old fruits. The basis of lower levels of PME activity, protein, and mRNA in *Nr* fruits as compared with *nor* fruits during the later stages of fruit maturation is not clear. However, this could be related

to the effects of these mutations on the overall development and ripening of mutant fruits.

Comparison of the effects of *rin, nor,* and *Nr* mutations on the expression of PME and PG genes during tomato fruit development provides further insight into the nature of these mutations. The *rin* mutation shows the greatest effect on the expression of PME, virtually eliminating PME mRNA by 55 DAF. The effect of the *rin* mutation on PME gene expression is similar to the effect observed on PG gene expression (2). In an earlier study (2), we showed that PG mRNA was detectable at a very reduced level in 45 DAF *rin* fruit, but not in the later stages of *rin* fruit development. Our data suggest that the *rin* mutation has an effect on the expression of several genes in developing fruits and begins to manifest itself by 35 DAF. Although the *nor* mutation also greatly depressed PG gene expression in tomato fruit (2), it does not impair the expression of the PME gene. The effect of the *Nr* mutation on temporal regulation of PME and PG gene expression at the mRNA levels is similar. Collectively, the results indicate that these mutations have different effects on the expression of genes involved in tomato fruit development.

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