Gene expression and enzymatic activity of pectin methylesterase during fruit development and ripening in *Coffea arabica* L.

S.M.B. Cação[1], T.F. Leite[1], I.G.F. Budzinski[1], T.B. dos Santos[1],
M.B.S. Scholz[1], V. Carpentieri-Pipolo[2], D.S. Domingues[1],
L.G.E. Vieira[1] and L.F.P. Pereira[3]

[1]Laboratório de Biotecnologia Vegetal, Instituto Agronômico do Paraná,
Londrina, PR, Brasil
[2]Departamento de Agronomia, Centro de Ciências Agrárias,
Universidade Estadual de Londrina, Londrina, PR, Brasil
[3]EMBRAPA Café, Londrina, PR, Brasil

Corresponding author: L.F.P. Pereira
E-mail: filipe.pereira@embrapa.br

ABSTRACT. Coffee quality is directly related to the harvest and post harvest conditions. Non-uniform maturation of coffee fruits, combined with inadequate harvest, negatively affects the final quality of the product. Pectin methylesterase (PME) plays an important role in fruit softening due to the hydrolysis of methylester groups in cell wall pectins. In order to characterize the changes occurring during coffee fruit maturation, the enzymatic activity of PME was measured during different stages of fruit ripening. PME activity progressively increased from the beginning of the ripening process to the cherry fruit stage. *In silico* analysis of expressed sequence tags of the Brazilian Coffee Genome Project database identified 5 isoforms of *PME*. We isolated and cloned a cDNA homolog of PME for further characterization. *CaPME4* transcription was analyzed in pericarp, perisperm, and endosperm tissues during fruit development and ripening as well as in other plant tissues.

Northern blot analysis revealed increased transcription of *CaPME4* in the pericarp 300 days after flowering. Low levels of *CaPME4* mRNAs were observed in the endosperm 270 days after flowering. Expression of *CaPME4* transcripts was strong in the branches and lower in root and flower tissues. We showed that *CaPME4* acts specifically during the later stages of fruit ripening and possibly contributes to the softening of coffee fruit, thus playing a significant role in pectin degradation in the fruit pericarp.

INTRODUCTION

Coffee is one of the most important agricultural commodities and is widely cultivated in tropical countries. Commercial production relies on two species, *Coffea arabica* L. and *C. canephora* Pierre ex Froehner, which represent about 70 and 30% of the total coffee market, respectively (Vieira et al., 2006). The "cup quality" of coffee is directly associated with the ripening stage of the fruits during harvesting (Montavon et al., 2003). Inadequate harvesting and post-harvesting practices, including the mixing of coffee berries at different ripening stages (i.e., green fruits and over-ripe fruits in the same batch of cherries), may negatively affect the quality of coffee by altering the acidity and bitterness (Rena et al., 1996). One possible strategy for obtaining fruits in the same stage of ripening is to use selective harvesting techniques; however, this is an expensive practice (Rena et al., 1996). Another approach is the use of coffee plants with uniform ripening, which would facilitate mechanical harvesting of high-quality beans (Ribas et al., 2006).

Fruit ripening is a highly coordinated and genetically programmed phenomenon that leads to the development of a fruit with specific desirable attributes (Prasanna et al., 2007). Fruit ripening is also associated with textural alterations, which are dramatic in climacteric fruits such as coffee (Pereira et al., 2005). The major textural changes that cause softening of fruit result from enzyme-mediated alterations in the structure and composition of the cell wall and partial or complete solubilization of cell wall polysaccharides, such as pectins (Prasanna et al., 2007).

Pectins, the major constituent of the primary cell wall in higher plants, are a heterogeneous group of polymers, of which homogalacturonan is the most important. They are synthesized in the Golgi apparatus and are secreted into the wall in a highly methylesterified state (reviewed by Micheli, 2001). Pectin methylesterases (PMEs) catalyze the removal of methyl esters from the linear homogalacturonan backbone within cell walls, which releases methanol and protons, thus remodeling its fine structure and affecting its susceptibility to subsequent enzymatic and non-enzymatic reactions (e.g., pectin depolymerization) and gel formation (Jolie et al., 2010).

PMEs are ubiquitous enzymes involved in various physiological processes associated with both vegetative and reproductive plant development. Several studies have shown a strong correlation between PME activity or PME gene expression and physiological processes, such as hypocotyl elongation (Derbyshire et al., 2007), fruit ripening and maturation (Tieman and Handa, 1994; Prasanna et al., 2007), cellular adhesion (Roberts et al., 2000), seed germination

(Ren and Kermode, 2000), cambial cell differentiation (Micheli et al., 2000), and cell growth in developing wood cells (Siedlecka et al., 2008).

PME isoforms are encoded by large multi-gene families in all plant species. On the basis of phylogenetic analyses, they can be classified into 8 major clades (Markovic and Janecek, 2004). Their enzymatic and/or transcriptional activity have been reported in several tissues: fruits, leaves, flowers, stems, and roots. Some isoforms are constitutively expressed throughout the plant, whereas others are tightly regulated in specific tissues (pollen, fruits, or roots) or developmental stages (fruit ripening, microsporogenesis, or germination) (Jolie et al., 2010).

During fruit softening, pectins and hemicelluloses typically undergo solubilization and depolymerization, which contribute to cell wall loosening and disassembly (Wakabayashi et al., 2000). Changes in the pectin matrix are important for regulating the cell wall structure during fruit ripening and senescence. In this context, PME activity constitutes a key control point for both the assembly and disassembly of pectin networks (Brummel et al., 2004).

Coffee fruits have a high percentage of pectins in their pericarp (Pimenta et al., 2000). Changes in PME activity associated with fruit maturation may affect harvesting practices and post-harvesting operations (drying, de-pulping, etc.) to directly affect cup quality. Therefore, the objective of this study was to investigate the role of PME during coffee fruit ripening. Accordingly, we identified PME isoforms using the available expressed sequence tags (ESTs) from the Brazilian Coffee Genome Project database. One PME isoform was selected (*CaPME4*) for detailed analysis of transcriptional activity during development and ripening of *C. arabica* fruits concomitantly with enzymatic activity.

MATERIAL AND METHODS

Search for *C. arabica* PME sequences

In silico identification of *C. arabica* putative PME unigenes followed similar steps as those described in Budzinski et al. (2011). ESTs coding for PME were selected from the database of the Brazilian Coffee Genome Project (http://www.lge.ibi.unicamp.br/cafe) using a key word search. This EST database contains 130,792 ESTs of *C. arabica*, 12,381 ESTs of *C. canephora*, and 10,566 ESTs of *C. racemosa*. They are distributed into 37 cDNA libraries sequenced from the 5'-end (Vieira et al., 2006; Mondego et al., 2011). After BLAST searches in the coffee EST database, all singlets and contigs of candidate PME unigenes were re-assembled using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). The unigenes were then compared with those in the NCBI database using BLASTX and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST; Altschul et al., 1997). The open reading frame (ORF) of each unigene was predicted using the ORF finder program (NCBI), and the deduced amino acid sequences were used to construct a phylogenetic tree to analyze the relationship between *CaPME*s and other full-length PME genes. The full-length *CaPME1*, *2*, *3*, *4*, and *5* cDNA nucleotide sequences were submitted to GenBank with the accession Nos. JN863078 (*CaPME1*), JN863079 (*CaPME2*), JN863080 (*CaPME3*), JN863081 (*CaPME4*), and JN863082 (*CaPME5*).

Phylogenetic classification

Five coffee PMEs with transcripts in fruit libraries were selected for analysis based

on the phylogenetic classification of PMEs proposed by Markovic and Janecek (2004). The tree with the highest log likelihood was inferred using the maximum likelihood method based on the Poisson correction model and 1000 bootstrap replicates. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Plant material

Fruits in different stages of ripening and other plant tissues (roots, branches, leaves, flower buds, and flowers) were collected from *C. arabica* cv. IAPAR-59 plants cultivated under field conditions at the Agronomic Institute of Paraná Experimental Station (Londrina, Brazil) from December 2006 to April 2007. Fruits were collected every 90 days after flowering until complete maturation and were separated by perisperm color as previously described (Budzinski et al., 2011). Unless otherwise stated, experiments were conducted at least twice with a minimum of 3 biological replicates per experiment. After harvesting, all samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction

To analyze *CaPME4* transcription patterns, total RNA from different plant organs and fruit tissues (pericarp, perisperm, and endosperm) at several developmental stages were extracted as described by Geromel et al. (2006). RNA quality was checked on 1% agarose gel electrophoresis, and total RNA quantification was performed using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

Isolation of a partial *CaPME4* cDNA clone

A partial sequence corresponding to *CaPME4* cDNA was amplified from mature whole fruit (cherry pericarp) for further use as a probe in Northern blot analysis. First-strand cDNA was synthesized from 1 µg total RNA using the ThermoScript™ RT-PCR System (Invitrogen) according to manufacturer instructions. To amplify the *CaPME4* ORF, specific primers were designed using Primer Designer 2.0: *CaPME* sense: 5'-CTC ATC GTT GGA AGT GGT GGT GG-3' and antisense: 5'-CTG TAA TCT CCG CTG CCA TCC T-3'. PCR was performed under the following conditions: initial denaturation at 94°C for 5 min; followed by 40 cycles of 94°C for 1 min, 60°C for 45 s, and 72°C for 1 min, and a final extension step of 72°C for 5 min using standard *Taq* Polymerase (Invitrogen, Brazil). Reaction products were analyzed on a 1% agarose gel. The amplified DNA fragment of 743 bp was cloned into pTOPO2.1 (Invitrogen) and inserted into *Escherichia coli* TOP10 cells. Plasmid DNA was prepared by alkaline lysis and column-purified (Qiagen, Chatsworth, CA, USA), and both strands of the cloned cDNA fragments were sequenced with a MegaBACE 1000 DNA Analysis System (GE Life Science, Pittsburg, PA, USA).

Northern blot analysis

Total RNA (15 µg) was denatured in 12.55 M formamide, 2.2 M formaldehyde, and

20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer at pH 7.0 (also containing 5 mM Na-acetate and 0.1 mM EDTA) at 65°C for 15 min and fractionated on a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde in 10X MOPS buffer. The RNA was transferred to nylon membranes by capillarity according to Sambrook et al. (1989). Membranes were pre-hybridized at 37°C for 2 h using UltraHyb® buffer (Ambion). The cDNA fragment of *CaPME4* was used as a template for probe synthesis. The probe was radiolabeled with [α-P^{32}]-dCTP by random priming, and membranes were hybridized at 37°C overnight in the same buffer. After hybridization, membranes were sequentially washed twice in 2X SSC with 0.1% SDS for 10 min at 42°C and twice in 0.1X SSC with 0.1% SDS for 10 min at 42°C before exposure to a BAS-IP MS 2340 imaging plate. Images were captured using a fluorescent image analyzer FLA 3000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

PME extraction

The PME extractions were carried out during 5 different stages of fruit ripening [120, 210, 240, 270, and 300 days after flowering (DAF)] from green to the full-ripe cherry in order to analyze the biochemical activity of PME in the pericarp. Tissue samples were extracted in 50 mM borate-acetate buffer at pH 7.5 with 0.1 M NaCl solution at 4°C. The ratio of coffee material to extractant was 1:3 (w/v). The homogenate was squeezed through two layers of gauze, and the extract was centrifuged for 10 min at 10,000 *g* to remove the solid particles (Körner et al., 1980). The supernatant was brought to 70% saturation by addition of solid ammonium sulfate and centrifuged at 10,000 *g* for 10 min after standing for 1 h. The precipitate was resuspended in borate-acetate buffer at a ratio of 1:3 (w/v).

Determination of PME activity

PME activity was measured titrimetrically by estimating the free carboxyl groups formed in pectin as a result of enzymatic action. The amount of 0.1 M NaOH required to maintain the median reaction at pH 7.5 (25°C) was measured by the method described by Kertesz (1955). The enzyme substrate was a 0.5% citrus pectin solution (Sigma) containing 0.15 M NaCl. One unit of PME was defined as the amount of enzyme that released 1 mmol carboxyl groups min$^{-1} \cdot$g^{-1} fresh weight of pericarp.

RESULTS AND DISCUSSION

In silico analysis

Data mining of the Brazilian Coffee Genome database (http://www.lge.ibi.unicamp. br/cafe/) yielded 162 candidate sequences that corresponded to annotated PME sequences. After local clustering, 5 contigs with ESTs were formed from fruit libraries, named *CaPME1* to *CaPME5* (Table 1). A similarity analysis of the selected cDNAs indicated that they were unique sequences representing different PME genes. After translation and conserved domain analysis, it was possible to identify 2 characteristic PME domains [Pfam01095 pectinesterase superfamily and Pfam04043 PMEI (inhibitor) superfamily]. *CaPME1* and *CaPME4* have both domains, while *CaPME2*, *CaPME3*, and *CaPME5* have only the domain, Pfam 01095. The

domain Pfam 01095 domain matches the pre-region that is required for protein targeting to the endoplasmic reticulum (Micheli, 2001; Dorokhov et al., 2006). Targeting to the endomembrane system leads to the export of PMEs into the cell wall and is mediated either by a common type signal peptide or a transmembrane domain (signal anchor). The pro-region (Pfam 04043) shares similarities with the PMEI domain and is thought to play an autoinhibitory role during the enzyme's secretion into the apoplast, where only the mature part of the protein is found (Giovane et al., 2004).

Table 1. Pectin methylesterase (PME) contigs with expressed sequence tags (ESTs) from fruit libraries.

PME	*Coffea arabica* contigs (Mondego et al., 2011)	FR ESTs (Mondego et al., 2011)*
CaPME1	Contig 521	3 FR ESTs/15 ESTs total
CaPME2	Contig 4211	1 FR ESTs/6 ESTs total
CaPME3	Contig 15241	1 FR ESTs/3 ESTs total
CaPME4	Contig 13700	5 FR ESTs/14 ESTs total
CaPME5	Contig 14456	4 FR ESTs/2 ESTs total

*Number of ESTs from fruit libraries/total ESTs from contig.

The deduced amino acid sequences of *C. arabica* and other known plant pectin methyesterases were aligned, and a phylogenetic tree was drawn to classify *Coffea* PMEs (Figure 1). Phylogenetic analysis showed that *Coffea* PMEs are distributed in 5 of the 9 clades assigned for plant PMEs (Markovic and Janecek, 2004). *CaPME1* (587 amino acids, aa) is classified into clade Plant1. This isoform has all characteristics described for this clade: a conserved segment, WPxWxxxxDRRLLQ, and a length between 520 and 590 residues. We identified all 5 conserved domains listed by Markovic and Janecek (2004), with only a single substitution in one residue of region III (Table 2). *CaPME2* (355 aa) is classified into clade Plant2a and contains the main features of this group: a conserved segment, PxWxxxxDRRLL, and one conserved histidine (His/H) preceding the mature enzyme. *CaPME2* has identical conserved regions in comparison to Q9M3B0_ARATH34 (Table 2), including a conserved segment, RR. *CaPME3* (273 aa) and other members of the Plant X2 clade do not contain the pre-pro region. This isoform also has a His residue that is conserved in PMEs of the Plant X2 clade and 4 of the 5 conserved PME regions (Table 2). *CaPME5* is grouped into clade Plant 1a, the largest plant clade (Markovic and Janecek, 2004). This PME has 325 residues and, although it is unusually small in length, contains all 5 conserved regions present in PMEs.

CaPME4 (582 aa) is classified into the clade Plant 3 that originally contained only 2 PMEs (Q43867_ARATH and Q43111_PHAVU). All features of this clade, such as typical length and 5 conserved regions, are observed in *CaPME4* (Table 2). Since this isoform was selected for further transcriptional analysis, details of its transmembrane domains and the pre- and pro-regions are depicted in Figure 2.

On the basis of the *in silico* analysis for identifying transcriptionally active PMEs in the CafEST database, we observed that *CaPME4* is the unigene that contains the largest number of reads from the fruit-specific cDNA library (5 reads, Table 1). Therefore, we chose this isoform for RNA analysis in order to gain insight into the transcription pattern of *CaPME* during coffee fruit development and maturation.
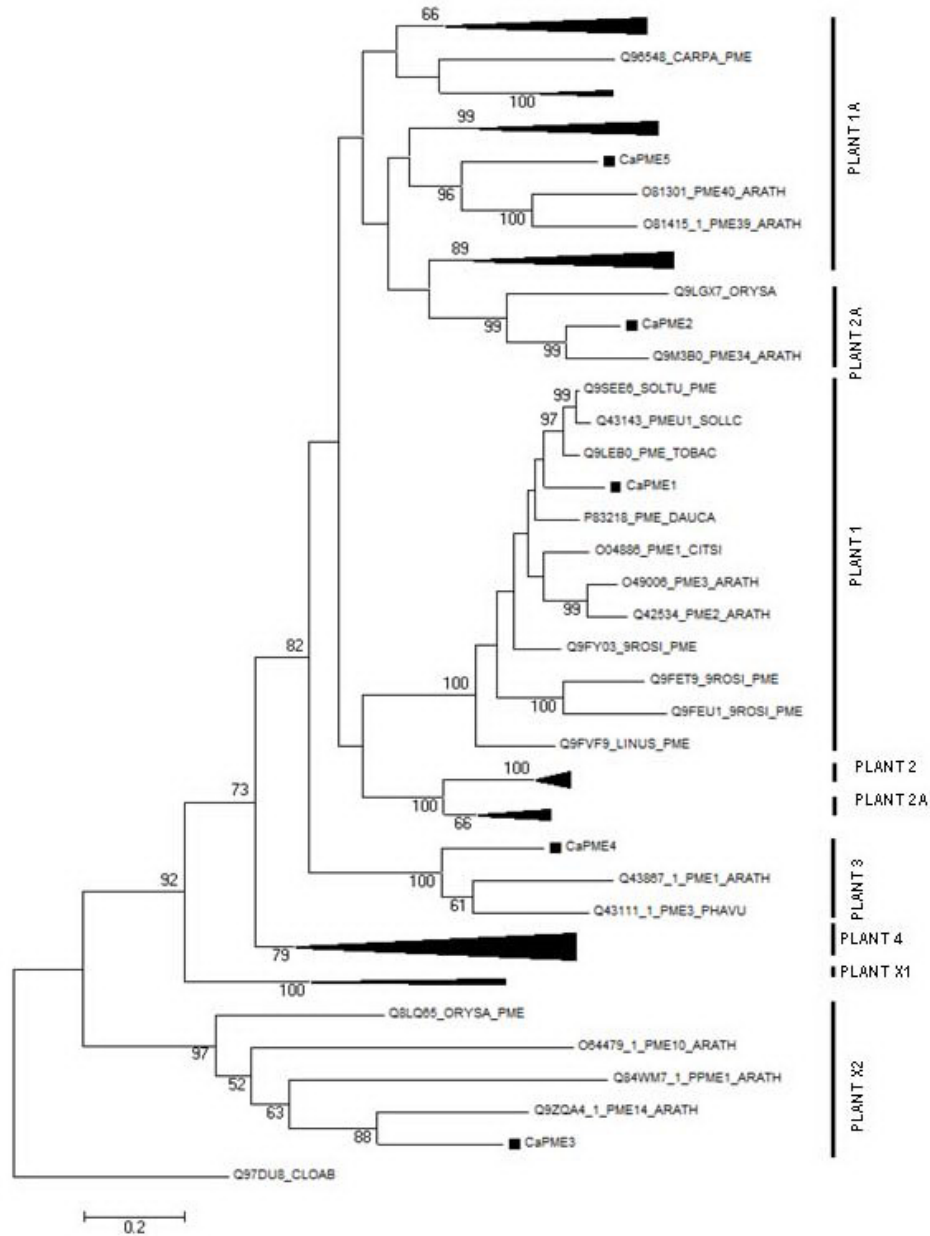
Figure 1. Phylogenetic analysis of *Coffea* pectin methylesterases (PMEs). The tree with the highest log likelihood was inferred by using the maximum likelihood method based on the Poisson correction model, and 1000 bootstrap replicates. Bootstrap values over 50% are shown. The analysis involved 48 protein sequences. All positions with less than 80% site coverage were eliminated. There were a total of 401 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Vertical lines indicate the 8 clades (Plant 1A, 2A, 1, 2, 3, 4, X1, and X2). Black boxes indicate coffee PMEs (*CaPME1*, *2*, *3*, *4*, and *5*) identified in the coffee database.

Table 2. Segments of conserved amino acid of *Coffea* pectin methylesterases (PMEs): *CaPME1* to *CaPME5*.

Clade	Accession/gene	Region I	Region II	Region III	Region IV	Region V
Plant1	P83218_DAUCA	94GVYREN	112HQVALR	134YQDTLYV	157DFIFG	223LGRPWK
	CaPME1	GVYREN	HQVALR	HQDTLYA	DFIFG	LGRPWK
Plant2a	Q9M3BO_ARATH34	320GKYEED	386HQAVALR	410YQDTLYV	433DFIFG	499LGRPWK
	CaPME2	GKYEED	HQAVALR	YQDTLYV	DFIFG	LGRPWK
Plantx2	Q8LQ65_ORYSA11	121GTYTEK	201KQAVALR	223AQDTLYD	246DFIFG	300LGRAWG
	CaPME3	Not found	AQAVALR	AQDTLND	DFIFG	LGRAWG
Plant3	Q43867_ARATH65	316GTYVEN	384HQAVAFR	406FQDTLYP	429DFIFG	490LGRPWK
	CaPME4	GVYKEK	HQAVAFR	FQDTLYP	DFIFG	LGRPWK
Plant1a	O04889_CITSI2	238GTYNEN	306HQAVALR	328YQDTLYV	351DFIFG	417LGRPWK
	CaPME5	GAYFEY	HQAVALR	YQDTLYV	DFIFG	LGRPWK

The segments are arranged into the clades: Plant1, 2a, x2, 3, 1a. The numbers in front of the segments indicate the position in the amino acid sequences based on Markovic and Janecek (2004) classification.
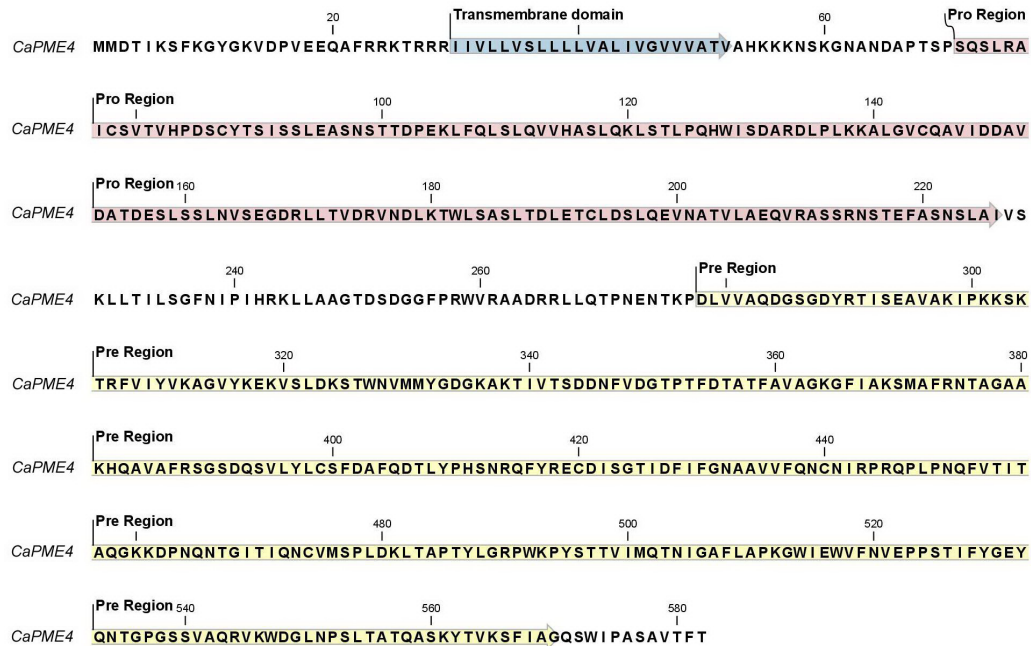

Figure 2. Predicted amino acid (aa) sequence of the *Coffea arabica* pectin methylesterase *CaPME4*. In blue is highlighted the transmembrane domain (18 aa); in red, the pro-region (156 aa) and in yellow pre-region (293 aa).

Expression of *CaPME4* genes in non-fruit tissues

Since PME is part of a multi-gene family, transcription in different plant tissues and stages of development as well as constitutive expression of some isoforms have been reported (Markovic and Janecek, 2004; Kim et al., 2010). Therefore, *CaPME4* transcript accumulation was investigated in the fully opened flower, flower bud, leaf, root, and young and old branches of *C. arabica* cv. IAPAR-59 (Figure 3). *CaPME4* transcripts were highly expressed in branches and roots with lower expression in flower buds. The presence of these transcripts

in branches and roots is probably due to cell-wall extension, cellular separation, internode stem growth, and root-tip elongation (Sobry et al., 2005).
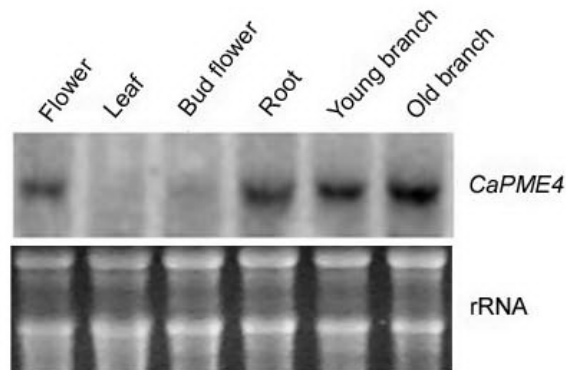
Figure 3. Northern blot of *CaPME4* in different coffee tissues. Total RNA stained with ethidium bromide was used to monitor the equal loading of the samples.

CaPME4 expression during fruit development and ripening and PME activity

We investigated the transcriptional pattern of *CaPME4* in coffee fruit tissues at different developmental and ripening stages in the pericarp (90-300 DAF), perisperm (90-150 DAF), and endosperm (90-300 DAF) (Figure 4). Expression of this isoform was observed in the pericarp only at 210 DAF, reaching a maximum at the final stages of ripening (300 DAF, cherry fruit). It is important to note that no transcriptional activity of *CaPME4* was detected in over-ripened fruits (dark cherry). At this stage, the coffee fruit is characterized by endosperm dehydration and alteration in pericarp color (De Castro and Marraccini, 2006). *CaPME4* signal was not observed in the perisperm, and a faint band was only detected at 270 DAF in the endosperm (seed).
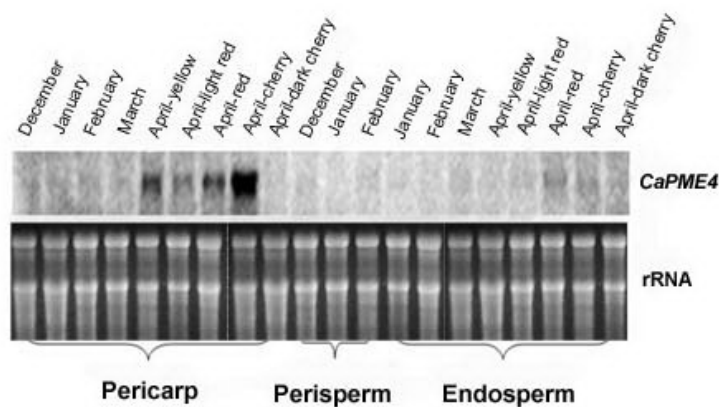
Figure 4. Northern blot of *CaPME4* during fruit development. Total RNA from the pericarp, perisperm and endosperm of *Coffea arabica* cv. IAPAR-59 from different stages of fruit development and ripening. Fruits were monthly collected: December to April. Different stages of fruit ripening were also collected during April according to fruit colors. December [90 days after flowering (DAF)], January (120 DAF), February (150 DAF), March (180 DAF), and April (210-300 DAF). Total RNA stained with ethidium bromide was used to monitor the equal loading of the samples.

PME activity increased progressively from the initial stage until the last stage of fruit ripening, which corresponds with the transcription analysis that showed maximum *CaPME4* mRNA expression in the pericarp at the cherry stage (Figures 4 and 5). The accumulation of early transcripts of one isoform of *PME* coupled with PME activity suggests that this *PME* is involved in cell expansion during the initial phase of development of the pericarp. PME activity before color change of the pericarp in coffee may also reflect signal induction in this climacteric fruit. Although PME is part of a large multi-gene family with functional redundancy, the isoform analyzed here is a strong candidate for further studies of coffee fruit development and ripening. Similar to our results, PME activity has been reported in several ripening climacteric fruits, including kiwifruit, papaya, avocado, and peach (Redgwell et al., 1990; Paull et al., 1999; Wakabayashi et al., 2000; Brummel et al., 2004).
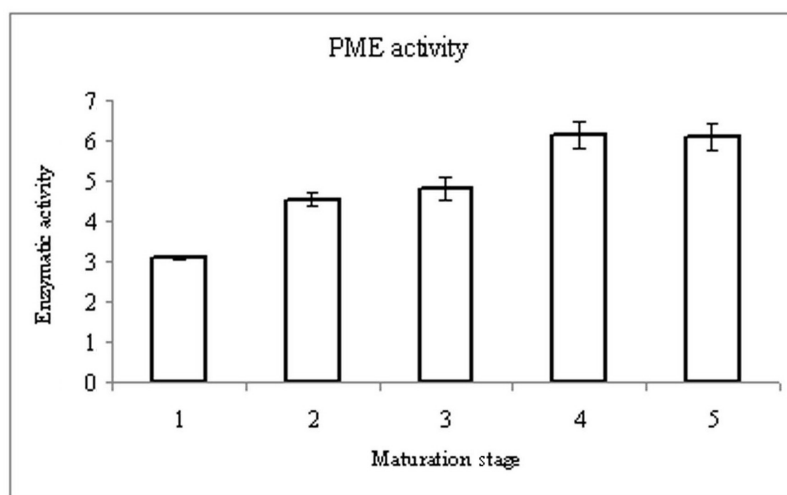
Figure 5. Specific activity of pectin methylesterases (PME) in different maturation stages of the coffee fruit, classified by the pericarp color: 1 - green [120 days after flowering (DAF)], 2 - light red (210 DAF), 3 - red (240 DAF), 4 - cherry (270 DAF), 5 - dark cherry (300 DAF). PME activity in mol of carboxyl groups in fresh weight pericarp $g^{-1} \cdot min^{-1}$.

During the ripening process, there are complex changes in the fruit wall due to the presence of cell wall softening enzymes and/or enzymatic inhibitors (Ali et al., 2004). Substantial synthesis of methyl-esterified pectin during the green stages and the increase in cell number and volume underlie the presence of high PME activity at early stages (Draye and Van Cutsem, 2008). PME catalyzes the hydrolysis of pectin methyl ester groups, resulting in de-esterification, which contributes directly or indirectly to the action of other enzymes by creating an appropriate ionic environment or possibly by modifying the cell wall porosity to increase the access of other enzymes to their substrates. PME preferentially attacks methyl ester bonds of galacturonate units next to non-esterified galacturonate units (Pilnik and Voragen, 1970), which precedes the activity of polygalacturonase (PG) (Koch and Nevins, 1989). In papaya, PME activity increased during maturation, which was followed by elevation of PG activity and decreased pulp texture (Lazan et al., 1995). In coffee fruits, PG activity is increased in the pericarp during the latest stages of ripening (Cação et al., 2003).

Coffee fruits have unusual tissue organization and development. The developing endosperm progressively replaces the perisperm inside locules; this process is almost complete by 120-150 DAF, while the remaining perisperm resembles a thin pellicle surrounding the endosperm. The storage phase, when cell walls of the endosperm begin to thicken due to the deposition of complex polysaccharides, occurs 130-190 DAF (De Castro and Marraccini, 2006). Towards the end of coffee fruit maturation, when endosperm development is complete, the final process of ripening is initiated. In climacteric fruits like coffee, a burst of ethylene occurs, followed by an increase in size and a change in color of the pericarp (Pereira et al., 2005). Our results showed an increased of both transcript level and enzymatic activity of PME during ripening (210-300 DAF) concomitant with development of the pericarp (Figures 4 and 5).

In this study, we showed that the *CaPME4* isoform acts specifically during the later stages of fruit ripening. This may contribute to coffee fruit softening, suggesting that *CaPME4* plays a significant role in pectin degradation in the fruit pericarp. As a result, the *CaPME4* gene could be an early molecular marker for color change of the pericarp and, consequently, for coffee fruit maturation.

ACKNOWLEDGMENTS

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