

## RESEARCH PAPER

# Expression patterns of three $\alpha$ -expansin isoforms in *Coffea arabica* during fruit development

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Coffee; expansin isoforms; fruit ripening; grain quality; perisperm.

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**ABSTRACT**

As a first step towards understanding the physiological role and regulation of the expansin gene (*EXP*) family in *Coffea arabica* fruits during growth and maturation, we identified 11 expansin genes, nine belonging to the  $\alpha$ -expansin family (*EXPA*), one *EXLA* and one *EXLB*, through *in silico* analysis of expressed sequence tags (ESTs). Within the  $\alpha$ -expansin family, three isoforms were selected for detailed examination based on their high expression in coffee fruits or because they were specifically induced during different fruit developmental stages, according to the EST information. The expression patterns were analysed in different fruit tissues (perisperm, endosperm and pericarp) of *C. arabica* cv. IAPAR-59 and *C. arabica* cv. IAPAR-59 Graúdo, the latter being a closely related cultivar with a larger fruit size. Accumulation of *CaEXPA1* and *CaEXPA3* transcripts was high in the perisperm (tissue responsible for coffee bean size) and in the early stages of pericarp development. Transcripts of *CaEXPA2* were detected only in the pericarp during the later stages of fruit maturation and ripening. There was no detectable transcription of the three *EXPs* analysed in the endosperm. The observed differences in mRNA expression levels of *CaEXPA1* and *CaEXPA3* in the perisperm of IAPAR-59 and IAPAR-59 Graúdo suggest the participation of these two isoforms in the regulation of grain size.

**INTRODUCTION**

Coffee is one of the most valuable traded commodities in the world, being cultivated in more than 60 countries. Commercially, coffee production relies only 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). Coffee quality has been associated with the ripening stages of fruits during harvest time, among other factors. In coffee growing regions with good rainfall distribution, flowering can occur over a long period. In this case, fruit growth is asynchronous during development, resulting in the presence of fruits at different ripening stages in the same plant. The presence of green fruits and over-ripe fruits in the same batch of grains changes the acidity, bitterness and, consequently, the quality of the beverage (Pereira *et al.* 2005). Besides the biochemical composition, coffee cup quality is also directly related to the structural features of the mature grain. The coffee grain size and its uniformity are important attributes for quality as well as for market price, since these characteristics are associated with higher prices according to international standards of classification.

In *C. arabica*, the time from anthesis to full ripening of fruits is 6–8 months (De Castro & Marraccini 2006). The *C. arabica* fruit is mainly composed of pericarp and perisperm tissues at the early stages of development (from

pollination up to 90 days after flowering – DAF). The perisperm undergoes intense cell division and expansion during these early stages of fruit development. This tissue is considered to be responsible for defining the final coffee grain size (De Castro & Marraccini 2006). During later stages of fruit development, the perisperm gradually disappears and is progressively replaced by the endosperm (true seed). Initially present in a 'liquid' state, the endosperm hardens as it ripens during the maturation phase as a result of the accumulation of storage proteins, sucrose and complex polysaccharides that represent the main seed reserves. The last step of maturation is characterised by dehydration of the endosperm and colour change of the pericarp (Eira *et al.* 2006). At this late ripening stage, the fruit starts climacteric development, with an ethylene burst and intense biochemical activity of cell wall loosening enzymes (Pereira *et al.* 2005). During fruit growth, cell enlargement is tightly linked to one or more loosening processes that modify the linkage of cellulose microfibrils to one another and to the cell wall matrix (Yong *et al.* 2007). Different proteins and enzymes act as agents of cell wall loosening, *e.g.*, expansins, xyloglucan endotransglycosylases/hydrolases and endo-(1,4)- $\beta$ -d-glucanases (for a review see Cosgrove 2005).

Expansins are plant cell wall loosening proteins that were first described in expanding/growing tissues as proteins that induce cell wall extension and stress relaxation at acidic pH (McQueen-Mason *et al.* 1992). It has been proposed that

expansins bind to the interface between microfibrils and polysaccharides and facilitate cell wall extension by catalysing the disruption of hydrogen bonds at the cellulose-hemicellulose interface, thereby facilitating turgor-driven slippage between microfibrils and other components of the cell wall (McQueen-Mason & Cosgrove 1995; Cosgrove 2000). These proteins play important roles in a range of developmental processes, including fruit softening (Brummell *et al.* 1999), pollination (Cosgrove *et al.* 1997), meristem dynamics (Fleming *et al.* 1997), root development (Zhang & Hasenstein 2000), cell enlargement, seed germination, abscission, stress responses and parasitism (reviewed by Choi *et al.* 2006). Expansin action during fruit development and ripening has been investigated in tomato (Rose *et al.* 1997; Brummell *et al.* 1999; Powell *et al.* 2003), peach (Obenland *et al.* 2003), fig (Omondi *et al.* 2004), mango (Sane *et al.* 2005) strawberry (Civello *et al.* 1999; Dotto *et al.* 2006), banana (Trivedi & Nath 2004; Asha *et al.* 2007) and grape (Ishimaru *et al.* 2006). Expansins are encoded by a multigene family and, based on sequence similarity, these proteins can be divided into four subfamilies, namely  $\alpha$ -expansin (EXPA),  $\beta$ -expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB).  $\alpha$ -Expansin and  $\beta$ -expansin have been demonstrated experimentally to cause cell wall loosening (Cosgrove *et al.* 1997; Li *et al.* 2003), whereas expansin-like A and expansin-like B are known only from their gene sequences (Sampedro & Cosgrove 2005). Expansins have a signal peptide containing about 20 amino acids at their N-terminus, which directs the polypeptide into the endoplasmic reticulum/Golgi secretory pathway (Lin *et al.* 2005). EXPAs and EXPBs have conserved cysteine residues in the N-terminal putative catalytic domain, a histidine-phenylalanine-aspartate (HPD) motif in the centre region and conserved tryptophan residues in the C-terminal putative cellulose binding domain (Choi *et al.* 2006).

Following previous studies on characterisation of genes and biochemical changes occurring during coffee fruit development and maturation (Geromel *et al.* 2006, 2008; Pereira *et al.* 2005; Salmona *et al.* 2008), the present work describes the transcription profile of three  $\alpha$ -expansin isoforms that are related to changes in grain size and to the final process of ripening of *C. arabica* fruits.

## MATERIAL AND METHODS

### Search for *C. arabica* expansin sequences

Expressed sequence tags (ESTs) coding for expansins were selected from the database of the Brazilian Coffee Genome Project (<http://www.lge.ibi.unicamp.br/cafe>) through a keyword search. This EST database contains 130,792 ESTs of *C. arabica*, 12,381 ESTs of *C. canephora* and 10,566 ESTs of *C. racemosa*, distributed into 37 cDNA libraries sequenced from the 5' end (Vieira *et al.* 2006). After local BLAST searches, all the singlets and contigs of candidate expansin unigenes were re-assembled using the software Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). The unigenes thus generated were compared with those in the NCBI database using the BLASTX program (<http://www.ncbi.nlm.nih.gov/BLAST>; Altschul *et al.* 1990). The open-reading frame (ORF) of each unigene was predicted using the ORF Finder program (NCBI). ScanProsite ([\[www.expasy.org\]\(http://www.expasy.org\)\) was used to verify the two domains specific for mature expansin proteins and to indicate the N-glycosylation linkage site. The pollen allergen domain was verified for each isoform using BLASTP. Signal peptide predictions were performed using SignalP version 3.0 \(<http://www.cbs.dtu.dk/services/SignalP>\). Using the deduced amino acid sequences, full-length proteins were aligned using CLUSTALW. The phylogenetic tree was constructed with MEGA – Molecular Evolutionary Genetics Analysis, version 3.1, software. The aligned sequences were used as the basis for construction of a comprehensive tree to verify similarity within the coffee expansin sequences and also to classify each expansin into a gene family \(EXPA, EXPB, EXP-like-A and EXP-Like-B\) using \*Arabidopsis thaliana\* \(At\), \*Medicago truncatula\* \(medi\), \*Gossypium hirsutum\* \(cot\), \*Solanum lycopersicum\* \(toma\) and \*Lactuca sativa\* \(let\), as recommended at the website <http://www.bio.psu.edu/expansins/naming.htm>: AtEXPA1 \(NM\\_105622\), AtEXPA2 \(U30481.3\), AtEXPA4 \(NM\\_129526\), AtEXPA6 \(NM\\_128454\), AtEXPA7 \(NM\\_101127\), AtEXPA8 \(NM\\_129623\), AtEXPA10 \(NM\\_102440\), AtEXPB1 \(NM\\_127640\), AtEXPB2 \(NM\\_105241\), AtEXPB3 \(NM\\_118965\), AtEXLA1 \(NM\\_114466\), AtEXLA2 \(NM\\_120002\), AtEXLA3 \(NM\\_001035738\), AtEXLB1 \(NM\\_117807\), medi\(TC88001\), cot1\(CD809349\), cot2\(TC21359\), toma \(TC118460\) and let\(TC982\). We also include sequences from \*Pyrus communis\* \(Pc\), \*Malus domestica\* \(Md\) and \*Musa acuminata\* \(Ma\): PcExp1 \(BAC67188.1\), PcExp2 \(BAC67189.1\), PcExp3 \(BAC67190.1\), PcExp4 \(BAC67191.1\), PcExp5 \(BAC67192.1\), PcExp6 \(BAC67192.1\), PcExp7 \(BAC67194.1\), MdEXPA3 \(BAD00013.1\), MaEXP1 \(AAM08930.1\), MaEXP2 \(AAN16378.2\), MaEXP3 \(ABM66430.1\), MaEXP4 \(ABN09939.1\) and MaEXP5 \(ABN09940.1\).](http://</a></p>
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The phylogenetic trees were inferred from a neighbor-joining tree using the Kimura two-parameter model. Bootstrap analysis was carried out (1000 trials) to assess support for individual nodes.

### Plant material

Fruits and other plant tissues (roots, plagiotropic shoots, leaves, flower buds and flowers) were harvested from *C. arabica* cv. IAPAR-59 plants, cultivated under field conditions at the experimental station of the Agronomic Institute of Paraná (Londrina, Brazil). Fruits were also collected from *C. arabica* cv. IAPAR-59 Graúdo, a closely related genotype having a larger fruit size (see Fig. S1 in the Supporting information section).

Fruits were collected monthly after flowering (end of September 2006) until complete maturation (end of April 2007). At the final stages of maturation (March and April), fruits were also classified based on pericarp colour (Table 1). After harvesting, all samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction. To observe spatial and temporal differences in expansin transcriptional activity, fruit tissues were dissected (pericarp, perisperm and endosperm) and used independently for RNA extraction.

### Genomic DNA and RNA extraction

Genomic DNA was extracted from young leaves of *C. arabica* cv. IAPAR-59 and cv. IAPAR-59 Graúdo with the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle

**Table 1.** *Coffea arabica* fruits grouped according to month and pericarp colour for molecular analysis.

month/year	days after flowering (DAF)	pericarp colour	developmental stage
October/2006	30	green	initial expansion
November/2006	60	green	(perisperm
December/2006	90	green	development)
January/2007	120	green	endosperm formation
February/2007	150	green	(grain filling)
March/2007 <sup>a</sup>	180	green-red <sup>a</sup>	fruit ripening
April/2007	210	yellow-cherry	

<sup>a</sup>Only green fruits were available from *C. arabica* cv. IAPAR 59 in March, whereas for *C. arabica* cv. IAPAR-59 Graúdo there were fruits with different pericarp colours.

1987). To analyse the expansin transcription patterns, total RNA from different plant parts and fruit tissues (pericarp, perisperm and endosperm) at different stages of development were extracted as described by Geromel *et al.* (2006, 2008). Total RNA quantification was performed using a Qubit<sup>™</sup> fluorometer (Invitrogen, Carlsbad, CA, USA).

#### Cloning and sequencing of coffee $\alpha$ -expansin isoforms

First-strand cDNA was synthesised from total RNA (1  $\mu$ g) using the ThermoScript<sup>™</sup> RT-PCR system (Invitrogen) according to the manufacturer's instructions. Based on sequences of the three selected  $\alpha$ -expansin contigs, specific primers were designed using the software Primer Designer version 2.0: *CaEXPA1* sense 5'-TTC CGA TGG CGT TCT CCT CC -3' and antisense 5'-GTC GTT AGT GAT AGG CAG T-3'; *CaEXPA2* sense 5'-GAG CAA GTG ACC GTC GCT CTT CCA CT-3' and antisense 5'-CTA TGA ATC ATG GTT CGA C-3'; *CaEXPA3* sense 5'-CGA GCG TAT TGC ACC TCC; antisense 5'-CAC GTT GCG GGG CAT ACA AT-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, 72 °C for 1 min and a final extension step of 72 °C for 5 min. Reaction products were analysed on a 1% agarose gel. The amplified DNA fragment obtained for each coffee expansin isoform was ligated in pTOPO2.1 (Invitrogen) and amplified in *E. coli* TOP10 cells. Plasmid DNA was prepared by alkaline lysis, column-purified (Qiagen, Chatsworth, CA, USA), and both strands of the cloned cDNA fragments were sequenced in a MegaBACE 1000 DNA Analysis System (GE Life Science, Pittsburgh, PA, USA).

The nucleotide sequences of the full-length *CaEXPA1*, 2 and 3 cDNAs described here have been submitted to GenBank and the assigned accession numbers are GQ434001, GQ434002 and GQ434003, respectively.

#### Northern and Southern blot analysis

Total RNA (15  $\mu$ g) was denatured in 12.55 M formamide, 2.2 M formaldehyde and 20 mM 3-(N-morpholino)-propane-sulphonic acid (MOPS) buffer, 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 10 $\times$  MOPS buffer. The RNA was transferred to nylon membranes by capillarity according to Sambrook *et al.* (1989). Membranes were pre-hybridised at 37 °C for 2 h using the buffer UltraHyb<sup>®</sup> (Ambion, Austin, TX, USA). The cDNAs of each  $\alpha$ -expansin were used as a template for probe synthesis. The probes were checked for specificity using a dot blot containing the nucleic acids of the three *EXPA* isoforms. Probes were labelled with [ $\alpha$ -P<sup>32</sup>]-dCTP using random priming, and the membranes were hybridised at 37 °C overnight in the same buffer. After hybridisation, membranes were washed twice for 10 min with 1 $\times$  SSC:0.1% SDS solution followed by washing twice for 10 min 1 $\times$  SSC:0.1% SDS. The membranes were exposed to film BAS-IP MS 2340. Images were captured using a fluorescence image analyser, FLA 3000 series (Fuji Photo Film CO, Ltd. Tokyo, Japan). For Southern blot analysis, 10  $\mu$ g DNA were digested with the restriction endonuclease *Hind*III (except for *CaEXPI* where *Dra*I was used), resolved on 0.8% (w/v) agarose gels, transferred to nylon membranes and hybridised with the same probes as described above.

## RESULTS

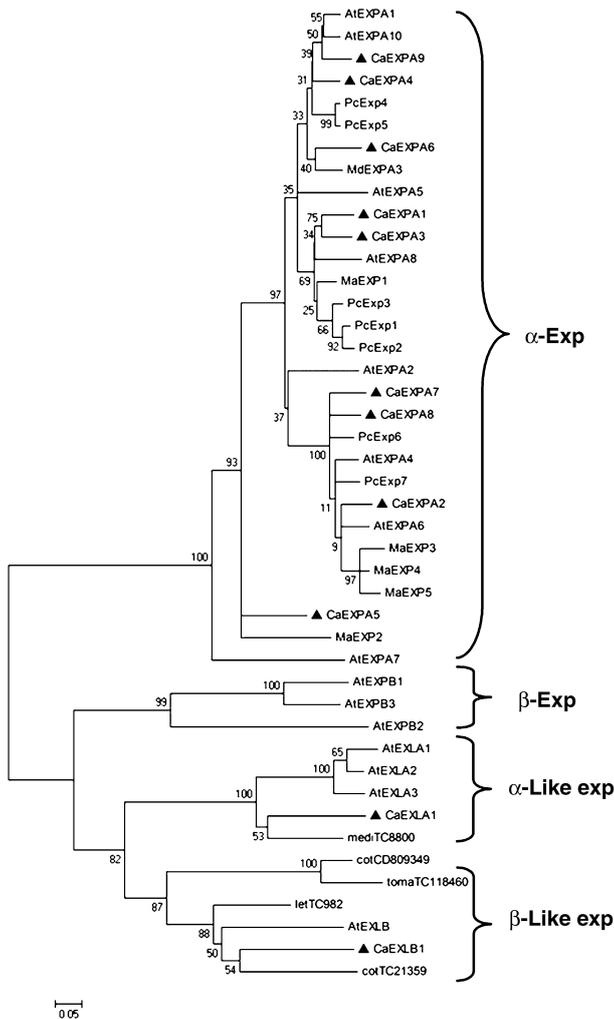
#### Coffee expansin identification and phylogenetic analysis

Data mining of the Brazilian Coffee Genome Project database (<http://www.lge.ibi.unicamp.br/cafe/>) yielded 11 candidate unigenes that corresponded to annotated expansin sequences. To confirm that all selected unigene clusters share similarities with expansins from other organisms, each consensus sequence was analysed at NCBI using BLASTX. DNA sequence analysis of the selected full-length cDNAs indicated that they were essentially unique sequences representing different expansin genes.

Based on analysis with the SignalP program, it was found that each coffee expansin protein had a cleavable signal peptide at the N-terminus. The length of this peptide varied, ranging from 18 to 31 residues. For all unigenes, there were two characteristic domains of expansin proteins: a cysteine-rich region (first domain) and a tryptophan-rich C-terminal portion (second domain). To classify the 11 coffee expansins within the four expansin families presently known ( $\alpha$ -EXP,  $\beta$ -EXP, EXLA and EXLB), a phylogenetic tree was generated from the alignment of the deduced amino acid sequences of the putative coffee expansins and expansins from *A. thaliana*, *O. sativa*, *S. lycopersicum*, *M. truncatula* and *L. sativa*, as recommended (<http://www.bio.psu.edu/expansins/naming.htm>; Fig. 1) Among the nine *C. arabica*  $\alpha$ -expansins identified from the phylogenetic tree, three were selected (*CaEXPA1*, *CaEXPA2* and *CaEXPA3*) for detailed examination based on the number of reads in coffee fruit cDNA libraries or their specific induction in fruits during different developmental stages.

#### Characteristics of *CaEXPA1*, *CaEXPA2* and *CaEXPA3* coffee $\alpha$ -expansins

The predicted proteins of *CaEXPA1* (257 amino acids), *CaEXPA2* (258 amino acids) and *CaEXPA3* (254 amino acids) provided the main features of this expansin subfamily, such as conserved cysteine (C) residues in the N-terminal region,



**Fig. 1.** Phylogenetic comparison of expansin protein sequences. Using the deduced amino acid sequences, full-length proteins were aligned using CLUSTALW. The phylogenetic tree was constructed using Mega version 3.1 software. Black triangles indicate coffee expansins. The other sequences represent different expansin families ( $\alpha$ -expansin,  $\beta$ -expansin, expansin-like A and expansin-like B).

an HPD domain (a catalytic site of the family 45-glycosyl hydrolases) and conserved tryptophan (W) residues at the C-terminal portion (Fig. 2). However, some variations specific to coffee expansins were also observed in the residues. For example, *CaEXPA1* had two W residues in the first domain (N-terminal) while *CaEXPA2* and *CaEXPA3* had three. In the second domain (C-terminal), *CaEXPA2* had five tryptophan residues while the other two isoforms had only four (Fig. 2).

#### Southern blot analysis

Southern blot analyses were carried out to determine the complexity of the three expansin genes in *C. arabica* cv. IAPAR-59 and cv. IAPAR-59 Graúdo (Fig. 3). Prior DNA blot analyses with different probes, under high stringency washing, revealed restriction fragments of different sizes for the three coffee expansins, indicating that the probes were gene-specific. Hybridisations with specific probes revealed two major

bands for the three coffee  $\alpha$ -expansin isoforms. This indicates that *CaEXPA1*, *CaEXPA2* and *CaEXPA3* are present in two copies. Hybridisation patterns of *CaEXPA1* and *CaEXPA2* in genomic DNA from cv. IAPAR-59 and cv. IAPAR-59 Graúdo were similar (Fig. 3). However, hybridisation with the *CaEXPA3* probe yielded a difference in band size between the two cultivars (Fig. 3).

#### Expression of *EXPA* genes during fruit development and ripening

To examine the potential role of  $\alpha$ -expansins in coffee fruit development and ripening, the hybridisation patterns of entire fruits with probes for *CaEXPA1*, *CaEXPA2* and *CaEXPA3* were evaluated at different developmental stages. Total RNAs from entire fruits were isolated each month (30–210 DAF) from cv. IAPAR-59 and cv. IAPAR-59 Graúdo. Transcript expression patterns of *CaEXPA1* and *CaEXPA3* were similar during the early stages of fruit development and in the final steps of fruit ripening for cv. IAPAR-59 (Fig. 4). Increased transcripts of these two isoforms were present at 60 DAF, a period of rapid fruit expansion and perisperm development (tissue responsible for defining final coffee grain size). In the genotype cv. IAPAR-59 Graúdo, we also observed transcripts of *CaEXPA1* and *CaEXPA3* at 60 DAF (Fig. 4), however there was a higher accumulation of mRNA at 90 DAF, which may indicate that, in this genotype, fruit expansion and perisperm development had continued until 90 DAF.

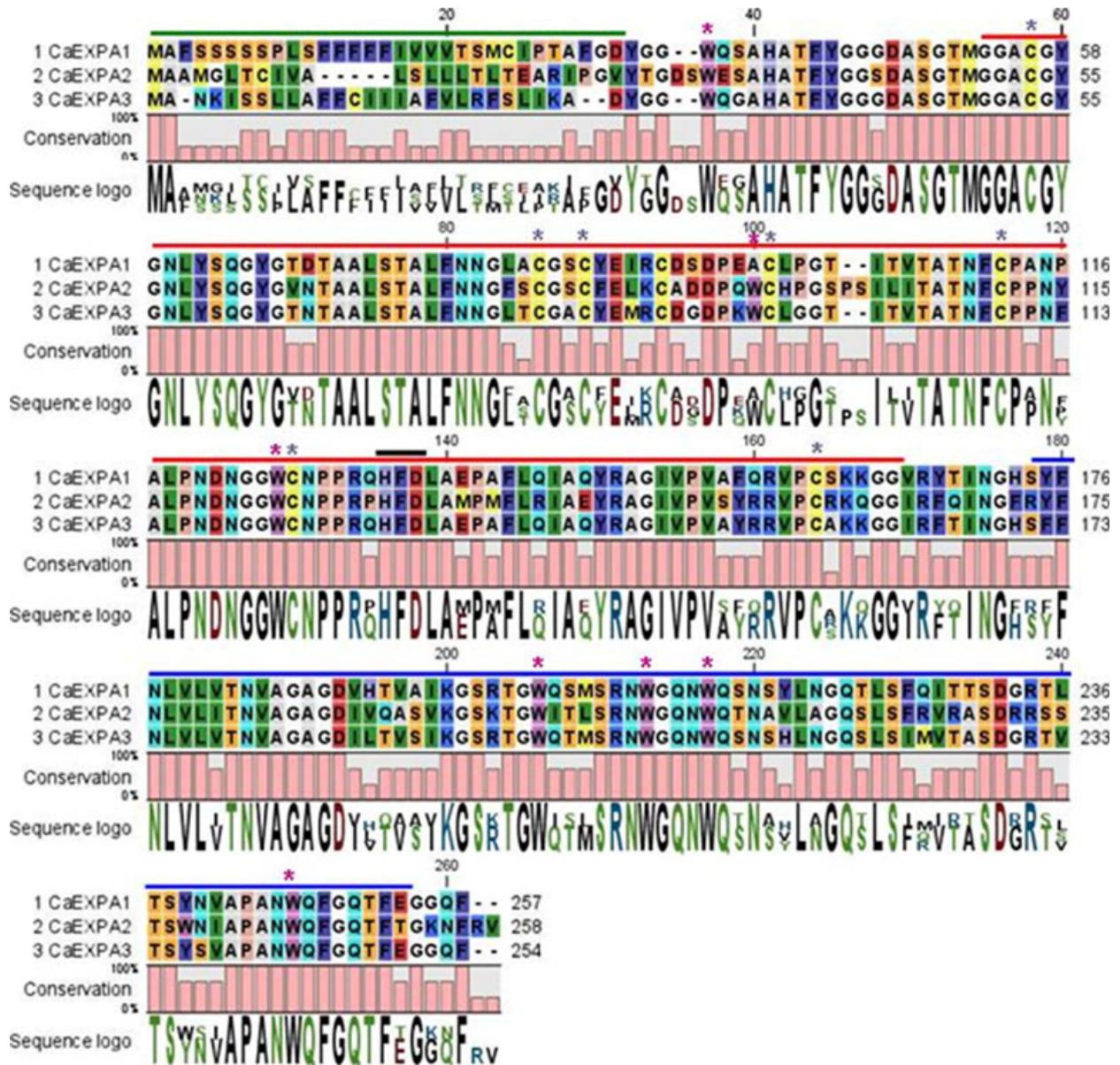
Increased transcripts of *CaEXPA1* and *CaEXPA3* were also observed at the final stages of ripening: April (210 DAF) for IAPAR-59 and March for IAPAR-59 Graúdo. These results suggest the participation of both isoforms in the fruit development and ripening process. In contrast, isoform *CaEXPA2* (Fig. 4) showed specific expression only during the latest stages of fruit ripening for both cultivars, at the time when the process of pericarp cell wall disassembly is initiated.

#### Expression of *EXPA* genes in individual fruit tissues

To examine differences in the transcription pattern of each isoform in different coffee fruit tissues, mRNA levels were evaluated individually for the pericarp (90–210 DAF), perisperm (90–150 DAF) and endosperm (90–210 DAF). For cv. IAPAR-59, transcription of *CaEXPA1* was observed in the pericarp from 90 DAF, reaching a maximum at 210 DAF stages: from yellow-red, light-red, red to cherry at 210 DAF; Fig. 5). In cv. IAPAR-59 Graúdo, increased expression of *CaEXPA1* was already observed in the pericarp at 90 DAF, the final step of rapid fruit expansion. Expression of this isoform was also observed in the pericarp at 180 DAF as well as in the final steps of ripening (210 DAF, April – cherry fruit; Fig. 5).

*CaEXPA3* showed the same transcription pattern as *CaEXPA1*, except for a smaller hybridisation signal in cv. IAPAR-59 at 210 DAF – cherry stage. *CaEXPA2* mRNA accumulation in the pericarp of IAPAR-59 and IAPAR-59 Graúdo coincided quite well with the pattern observed for the whole fruit (Fig. 4), with a hybridisation signal detected only during the ripening stages.

The perisperm tissue was already reduced to a thin membrane surrounding the endosperm at the beginning of fruit ripening (from 180 DAF onward), which hindered extraction



**Fig. 2.** Alignment of the deduced amino acid sequence of three coffee  $\alpha$ -expansins. Green bar indicates the signal peptide predicted by the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>). The potential putative catalytic domain (N-terminal) is indicated by a horizontal red bar and the putative cellulose binding domain (C-terminal) is indicated by a horizontal blue bar, both were predicted using ScanProsite. Purple asterisks (\*) = conserved tryptophan (W) residues; grey asterisks = conserved cysteine (C) residues. Multiple alignment was done with the CLC Workbench software.

of RNA for Northern blot analysis. *CaEXPA1* expression was observed in all perisperm tissues analysed from both cv. IAPAR-59 and cv. IAPAR-59 Graúdo, but at a much higher intensity at 90 DAF (Fig. 6). *CaEXPA2* expression was not observed in the perisperm (data not shown). Weak *CaEXPA3* transcriptional activity was observed in the perisperm of both cultivars only at 90 DAF (Fig. 6). No transcription was detected in the endosperm for any of the three *EXPA* isoforms tested (data not shown).

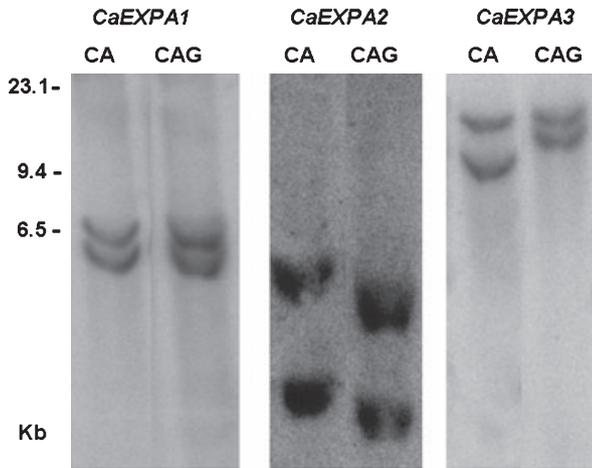
#### Expression of *EXPA* genes in non-fruit tissues

*CaEXPA1*, *CaEXPA2* and *CaEXPA3* mRNA accumulation was also investigated in roots, young and old branches, flower

buds, flowers and leaves of *C. arabica* cv. IAPAR 59 (Fig. 7). Detection of *CaEXPA1* and *CaEXPA3* transcripts was strong in flower buds, which are tissues with a high level of cell division. Transcripts of *CaEXPA1* were also observed at lower intensities in roots and young branches. *CaEXPA2* mRNA levels were low in roots, young and old branches and barely detectable in flower buds.

#### DISCUSSION

Coffee fruits have unusual tissue organisation and development. In *C. arabica*, the perisperm constitutes the predominant tissue occupying the inner space (locule) of fruits until 90–100 DAF. This locule space is delimited by the endocarp,

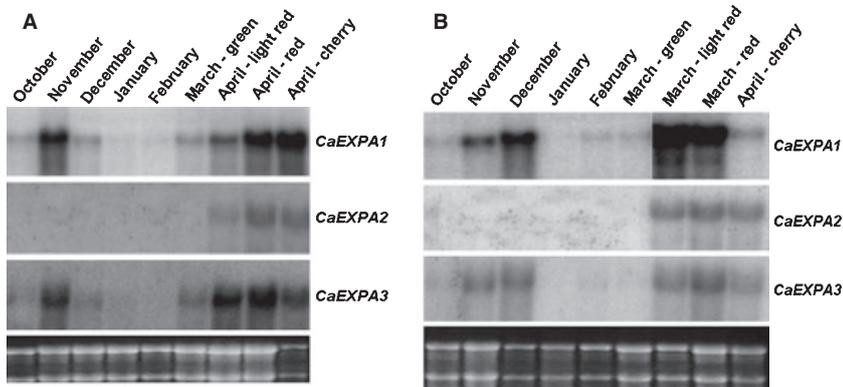


**Fig. 3.** DNA gel blot analysis. Genomic DNA from cv. IAPAR-59 and IAPAR-59 Graúdo were digested with the restriction enzymes *DraI* (*CaEXPA1*) or *HindIII* (*CaEXPA2* and *CaEXPA3*) and hybridised with probes from the cDNAs of each  $\alpha$ -expansin.

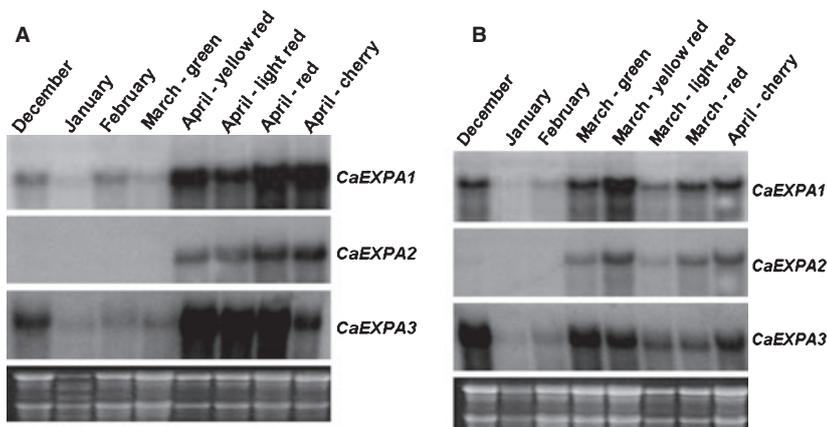
which is a hard and cellulose-rich tissue present from the early stages of fruit development (De Castro & Marraccini 2006). With growth of the fruit, this space is rapidly occupied by the endosperm, while the perisperm (at this point referred to the silver skin) becomes a thin tissue reduced to few cell layers surrounding the endosperm (Geromel *et al.*

2006, 2008). Therefore, the space occupied by the perisperm determines the final grain size. Towards the end of coffee fruit maturation, when endosperm development is complete, the final process of ripening is initiated. Similar to other climacteric fruits, a burst of ethylene occurs, followed by an increase in size and change in colour of the pericarp (Pereira *et al.* 2005). The expansin gene family is involved in different steps of fruit development and there are several reports on their temporal and spatial gene activity (Rose *et al.* 1997; Hayama *et al.* 2006; Ishimaru *et al.* 2006; Asha *et al.* 2007). Given that expansins may have a relevant role in perisperm expansion and fruit maturation, the main objective of this study was to analyse the tissue specificity and temporal expression pattern of three  $\alpha$ -expansin isoforms during coffee fruit development.

Initially, we identified 11 *C. arabica* expansin clusters within the Brazilian Coffee Genome Project database. All the hypothetical proteins exhibit specific functional motifs and domains that are characteristic of the expansin family. Based on phylogenetic analysis, these clusters were classified into nine  $\alpha$ -expansins, one expansin-like A and one expansin-like B. In *Arabidopsis*, 36 expansin genes have been reported (26  $\alpha$ -expansins, six  $\beta$ -expansins, three expansins-like A and one expansin-like B), while 58 expansins have been identified in rice (34  $\alpha$ -expansins, 19  $\beta$ -expansins, four expansin-like A and one expansin-like B) (Sampedro *et al.* 2005). The  $\beta$ -expansin family is more common in monocotyledons than in dicotyledons (Cosgrove 2000), which could explain the

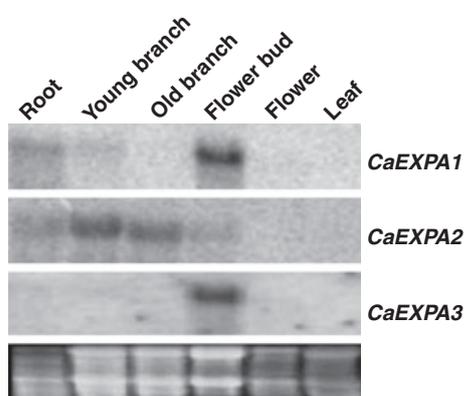
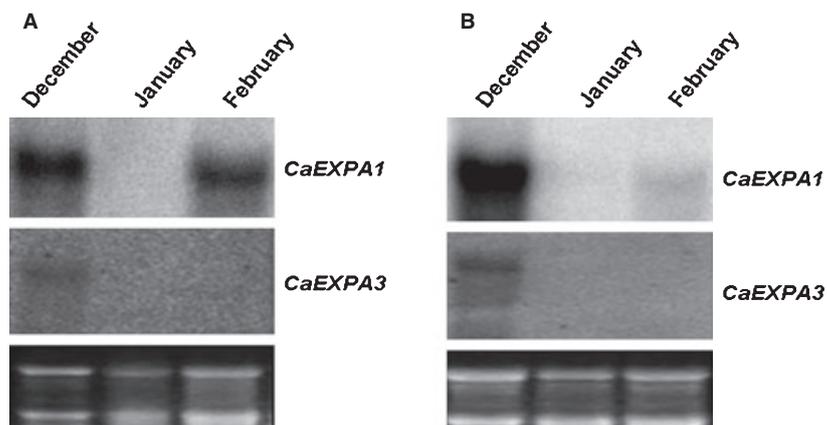


**Fig. 4.** Expression of *CaEXPA1*, *CaEXPA2* and *CaEXPA3* during coffee fruit development and ripening. Total RNA (15  $\mu$ g) was isolated from whole fruits of cv. IAPAR-59 (A) and cv. IAPAR-59 Graúdo (B). Fruits were collected each month after flowering: October (30 DAF), November (60 DAF), December (90 DAF), January (120 DAF), February (150 DAF), March (green, light-red, red, 180 DAF) and April (light-red, red and cherry, 210 DAF). Ethidium bromide-stained gel prior to blotting is shown below the blot as a loading control.



**Fig. 5.** Expression of *CaEXPA1*, *CaEXPA2* and *CaEXPA3* in the pericarp during coffee fruit development and ripening. Total RNA (15  $\mu$ g) was isolated from the pericarp of cv. IAPAR-59 (A) and cv. IAPAR-59 Graúdo (B). Fruits were collected each month after flowering: December (90 DAF), January (120 DAF), February (150 DAF), March (green, yellow-red, light-red and red, 180 DAF) and April (light-red, red and cherry, 210 DAF). Ethidium bromide-stained gel prior to blotting is shown below the blot as a loading control.

**Fig. 6.** Expression of *CaEXPA1* and *CaEXPA3* during coffee fruit development and ripening. Total RNA (15 µg) was isolated from the perisperm of cv. IAPAR-59 (A) and cv. IAPAR-59 Graúdo (B). Fruits were collected each month after flowering: December (90 DAF), January (120 DAF), February (150 DAF). Ethidium bromide-stained gel prior to blotting is shown below the blot as a loading control.



**Fig. 7.** Organ specific expression of *CaEXPA1*, *CaEXPA2* and *CaEXPA3* genes. Total RNA (15 µg) was extracted from roots, young branches, old branches, flower buds, flowers and leaves of *C. arabica* cv. IAPAR 59. Ethidium bromide-stained gel prior to blotting is shown below the blot as a loading control.

absence of this subgroup in the coffee database. Additional information on *Coffea* sequence data might reveal more expansins, including some from the  $\beta$  family.

Three  $\alpha$ -expansin cDNAs, *CaEXPA1*, *CaEXP2* and *CaEXPA3*, were cloned for further molecular characterisation. *CaEXP1* and *CaEXP3* showed higher homology than the *CaEXP2* isoform. The first two cDNAs were grouped into the same clade of the phylogenetic tree and shared a very similar transcription pattern (Figs 4 and 5), as discussed below. Asha *et al.* (2007) identified five banana expansins (*MaEXPA1*–*MaEXPA5*); of these *MaEXPA3*, *MaEXPA4* and *MaEXPA5* showed high sequence homology and clustered together in the phylogenetic tree. However, despite the high homology, they differed in their expression patterns.

A dot-blot analysis was first used to test the specificity of each probe and no cross-hybridisation was observed (data not shown). Southern blot analysis of these three Arabica coffee  $\alpha$ -expansins revealed unique patterns for each probe (Fig. 3). The restriction signals of *CaEXPA1*, *CaEXPA2* and *CaEXPA3* suggest that these genes are encoded as two copies in *C. arabica*. Since *C. arabica* is an allotetraploid, each parental species (*C. canephora* and *C. eugenoides*) can contribute one homeologous gene. The Southern hybridisation

signals for *CaEXPA1* and *CaEXPA2* were similar in cv. IAPAR-59 and cv. IAPAR-59 Graúdo. However, the observed difference in the band pattern of *CaEXPA3* suggests that the genomic structure of this isoform differs in these two genotypes (Fig. 3).

Northern blot analyses were initially used to observe transcriptional expression of each  $\alpha$ -expansin isoform in different non-fruit coffee tissues (Fig. 7). Other than fruits, *CaEXPA1* and *CaEXPA3* isoforms showed high mRNA accumulation in flower buds. On the other hand, *CaEXPA2* showed mRNA expression in roots, young and old branches, and at a comparatively low level in flower buds (Fig. 7). The presence of these transcripts in flower buds is probably due to the intense cell expansion and elongation that occurs in this tissue. In Kyoho grape, Ishimaru *et al.* (2006) reported the identification and characterisation of three  $\alpha$ -expansin cDNAs. From these, *VIEXP1* and *VIEXP2* were expressed at high levels in leaves, tendrils, roots, flowers and seeds, while *VIEXP3* was fruit-specific. Differential transcriptional activity of *EXP* isoforms in plant organs has also been reported in *Arabidopsis* (Bibikova *et al.* 1998; Cho & Cosgrove 2002), maize (Zhang & Hasenstein 2000), soybean (Lee *et al.* 2003) and *Rumex palustris* (Colmer *et al.* 2004).

During development and ripening, fruits undergo various changes in colour, texture, aroma and taste. Expansins are known to participate in these processes and are also believed to prepare the cell wall for subsequent degradation by cell wall hydrolases during ripening, particularly in climacteric fruits (Trivedi & Nath 2004). To characterise expression patterns of the three  $\alpha$ -expansin genes at various stages of coffee fruit development, RNA gel blot analysis was also performed using mRNA extracted from whole coffee fruit at different stages of development. *CaEXPA1* and *CaEXPA3* transcription patterns were similar in the whole fruit of *C. arabica* (Fig. 4). Expression of both isoforms was observed in the early stages of fruit development and in the later stages of ripening. It is tempting to speculate that the prolonged transcription of *CaEXPA1* and *CaEXP3* until 90 DAF in IAPAR-59 Graúdo (Fig. 5), a genotype characterised by larger fruits than IAPAR-59, is involved in modulation of fruit size. At this stage of development (30–90 DAF), the perisperm undergoes intense cell division, elongation and cell wall softening (De Castro & Marraccini 2006).

Differential expansin expression during fruit development and ripening has also been reported in several different fruits. For example, Hiwasa *et al.* (2003) isolated seven expansin cDNAs from pear (*PcExp1–PcExp7*) and characterised their expression patterns at various fruit development stages. The pear expansins were separated in three categories according to their unique expression profiles: (i) up-regulated at ripening, (ii) expressed but with no significant change during ripening and (iii) undetectable in ripening fruit. In Kyoho grape berries Ishimaru *et al.* (2006) observed essentially two expression patterns: increased expression with berry development up to the half-coloured stage and decreased until the mature stage (*Vlexp1* and *Vlexp3*), and increased expression before véraison, peaking at véraison and then remaining at high levels up to the maturation stage (*Vlexp2*). The expression pattern observed for *CaEXPA1* and *CaEXPA3* in the whole fruit was very similar to that observed in grapes for *Vlexp1* and *Vlexp3*. *CaEXPA1* and *CaEXPA3* had increased expression during the rapid fruit expansion stage and during pericarp colour change. In contrast, *CaEXPA2* increased its expression after pericarp colour change in the final steps of fruit maturation.

In order to assess the contribution of individual tissues to transcription levels of each  $\alpha$ -expansin isoform, *C. arabica* fruits were separated into endosperm, perisperm and pericarp. *CaEXPA1* and *CaEXPA3* transcription patterns detected in the pericarp were similar to those observed in whole fruit. After rapid fruit expansion (90 DAF), transcript levels of these two isoforms were down-regulated, and started to increase again at the ripening stages, a phase in which intense cell wall elongation and disassembly occurs in the pericarp. The simultaneous expression of both *CaEXPA1* and *CaEXPA3* may be important for cell wall loosening during coffee fruit development and for ripening, as these two  $\alpha$ -expansins may act synergistically during pericarp softening in the final stages of maturation. Another possibility is that these two  $\alpha$ -expansins act on different components of the cell wall (Im *et al.* 2000).

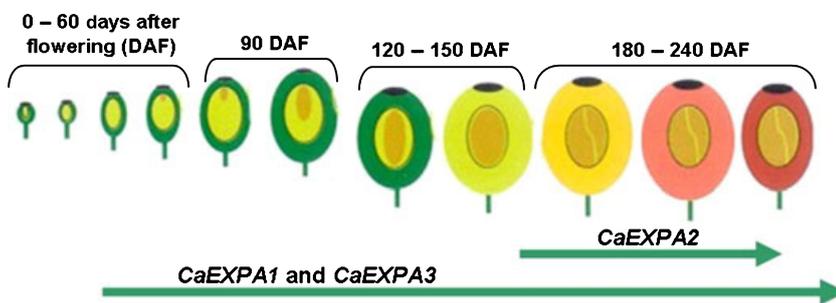
In IAPAR-59 Graúdo, the transition time among green and yellow-green fruits occurs early (at 180 DAF), the phase when we observed high mRNA accumulation for *CaEXPA1* and *CaEXPA3*; however the transition time in IAPAR-59 occurs between 180 DAF and 210 DAF. The highest level of both mRNA forms in fruits harvested at 180 DAF for IAPAR-59 Graúdo (Fig. 5) indicates that processes related to fruit ripening might have occurred earlier in the pericarp of this cultivar. Differential expression of expansins in apple pericarp during fruit growth was also observed by Kasai *et al.* (2008). The expression pattern of isoform (*MdEXPA3*) was

related to the growth rate of apple fruit, suggesting that this may control pericarp enlargement by regulating cell expansion. In our study, despite the difference in the time course, the transcription pattern of the three  $\alpha$ -expansins seems to be related to the same process of pericarp expansion in both IAPAR-59 and IAPAR-59 Graúdo.

About 90 days after flowering, the perisperm undergoes a process of rapid cell wall division and expansion, which in turn determines the physical limit of the space that will be occupied by endosperm. The highest transcription of *CaEXPA1* in cv. IAPAR-59 Graúdo at 90 DAF, compared to the small grain in cv. IAPAR-59 (Fig. 5), suggests possible involvement of *CaEXPA1* in the modulation of final fruit size in cv. IAPAR-59 Graúdo. Although *CaEXPA3* transcription in the perisperm was low, we cannot discard a supplemental role for this isoform in perisperm formation. Further research using RTq-PCR will help to clarify the roles of *CaEXPA1* and *CaEXPA3* in perisperm development. As the Southern blot analyses using a *CaEXPA3*-specific probe showed distinct hybridising bands in the two cultivars, it will be interesting to assess if the size polymorphism observed in one of the bands can be related to the differences in grain size. Further analyses of the genomic organisation of all three  $\alpha$ -expansins in these two cultivars are underway to provide additional information for understanding the role of these genes in coffee fruit development.

In climacteric fruits, such as coffee, ethylene is considered to be the trigger for fruit ripening events (Pereira *et al.* 2005). Due to the role of expansins in fruit ripening, various research groups have associated the modulation of expansin expression with ethylene (Rose *et al.* 1997; Hayama *et al.* 2006; Asha *et al.* 2007; Jackson 2008). *CaEXPA2* hybridisation with mRNA from the whole coffee fruit and the pericarp indicates the specific action of this isoform only in the final steps of fruit ripening in both IAPAR-59 and IAPAR-59 Graúdo, when there is increased production of ethylene (Pereira *et al.* 2005). In addition, specific expression in the pericarp and absence of transcripts in the perisperm and endosperm, suggests that *CaEXPA2* expression could be modulated by ethylene. This coffee  $\alpha$ -expansin isoform shows a similar mRNA expression pattern to the fruit-specific and ripening-related expansins *MaEXPA1* (Trivedi & Nath 2004) and *MaEXPA2* (Asha *et al.* 2007) in banana. Both of these genes are expressed abundantly only in mature fruit during ethylene-induced ripening and are repressed in fruit treated with 1-MCP (1-methylcyclopropene, an ethylene perception inhibitor).

Here, we present the first study on expression of  $\alpha$ -expansins in different tissues during coffee fruit ontogeny. The



**Fig. 8.** Hypothetical transcription pattern of *CaEXPA1*, *CaEXPA2* and *CaEXPA3*  $\alpha$ -expansin isoforms during coffee fruit development and ripening. Illustration adapted from De Castro & Marraccini (2006).

three  $\alpha$ -expansin isoforms examined showed differences in spatial and temporal transcription patterns (Fig. 8). As expansins might operate on different components of the cell wall, substrate specificity might explain the different expression of  $\alpha$ -expansins in coffee fruit tissues described here. While *CaEXPA1* and *CaEXP3* expression was mainly at stages when the coffee grain size is defined, *CaEXPA2* specificity to the pericarp indicates a role for this isoform during the ripening process (Fig. 8). Even taking into account that  $\alpha$ -expansins are encoded by a large multigene family with functional redundancy, the three isoforms presented here are attractive candidates for manipulating coffee fruit development and ripening.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Differences in fruit size between *C. arabica* cv. IAPAR-59 and *C. arabica* cv. IAPAR-59 Graúdo. Black bar = 1 cm.

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