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Characterization and Expression of Two cDNA Encoding 3-Hydroxy-3-methylglutaryl coenzyme A Reductase Isoforms in Coffee (*Coffea arabica* L.)

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Abstract

In higher plants there are two independent pathways for isoprenoid biosynthesis, located in the cytosol (mevalonic acid or MVA pathway) or in the plastids [methylerythritol phosphate (MEP) pathway]. The 3-hydroxy-3-methyglutaryl-CoA reductase (HMGR) is the first committed step in the MVA pathway. Using the information available from the Brazilian Coffee Genome Project, we found 13 ESTs that originated two isoforms, *CaHMGR1* and *CaHMGR2*, for the enzyme HMGR of *Coffea arabica*. A complementary DNA encoding the isoform *CaHMGR1* was cloned, and its complete nucleotide sequence determined. The full-length cDNA of *CaHMGR1* was 2,242 bp containing a 1,812-bp ORF encoding 604 amino acids. Bioinformatic analyses revealed that the deduced CaHMGR1 had extensive homology with other plant HMGRs and contained two transmembrane domains and two putative HMGR binding sites and two NADP(H)-binding sites. Under normal growth conditions, transcripts of isoform *CaHMRG1* were detected in fruit tissues (pulp, perisperm, and endosperm) only at the initial stages of development, flower buds and leaves. *CaHMRG2* was expressed in all tissues and during all fruit development stages examined. These results suggest a constitutive expression of isoform *CaHMGR2*, while the isoform *CaHMGR1* shows temporal and tissue-specific transcriptional activation.

Introduction

Coffee is one of the most important world agricultural commodities. It is consumed for more than 800 million people (Illy, 2006). The genus *Coffea* contains about 100 species but commercial production is based only on species *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) that represent 70 and 30% of the total coffee market, respectively (Vieira et al., 2006). Although the cup quality is one of the main aspects for coffee consumption, little is known about several components that can contribute for quality (Leroy et al., 2006).

Arabica and Robusta species present differences in the contents of caffeine, trigonelline and chlorogenic acids as well as in the amount and composition of lipids. Lipids are important components of coffee beverage and aroma. Coffee oil is composed mainly of triacylglycerols. The relatively large unsaponifiable fraction is rich in diterpenes of the kaurane family, mainly cafestol ($C_{20}H_{28}O_3$) and kahweol ($C_{20}H_{26}O_3$), which have been receiving increasingly attention in recent years due to their physiological effects in human health (Speer and Kölling-Speer, 2006).

Cafestol and khaweol originated from the isoprenoid pathway. Isoprenoids are the most functionally and structurally varied group of plant metabolites. Isoprenoids are synthesized in all organisms but are especially abundant and diverse in plants, with more than 25,000 compounds reported to date in the plant kingdom (Croteau et al., 2000; Rodriguez-Conception 2010; Sacchettini and Poulter, 1997). Despite their diversity in functions and structures, all isoprenoids derive from the common-five carbon-building unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Rodrigues-Conception and Boronat, 2002). In higher plants, two independent pathways located in separate intracellular compartments are involved in the biosynthesis of IPP and DMAPP (Fig. 1). In the cytosol, IPP is derived from the mevalonic acid (MVA) pathway (Chappell, 1995; Newman and Chappell, 1999; Qureshi and Porter, 1981), whereas in plastids, IPP is formed from pyruvate and glyceraldehyde 3-phosphate through the methylerythritol phosphate (MEP or nonmevalonate) pathway (Eisenreich et al., 1998; Rohmer, 1999). In the MVA pathway three molecules of acetyl-CoA condense successively to form 3-hydroxy-3-

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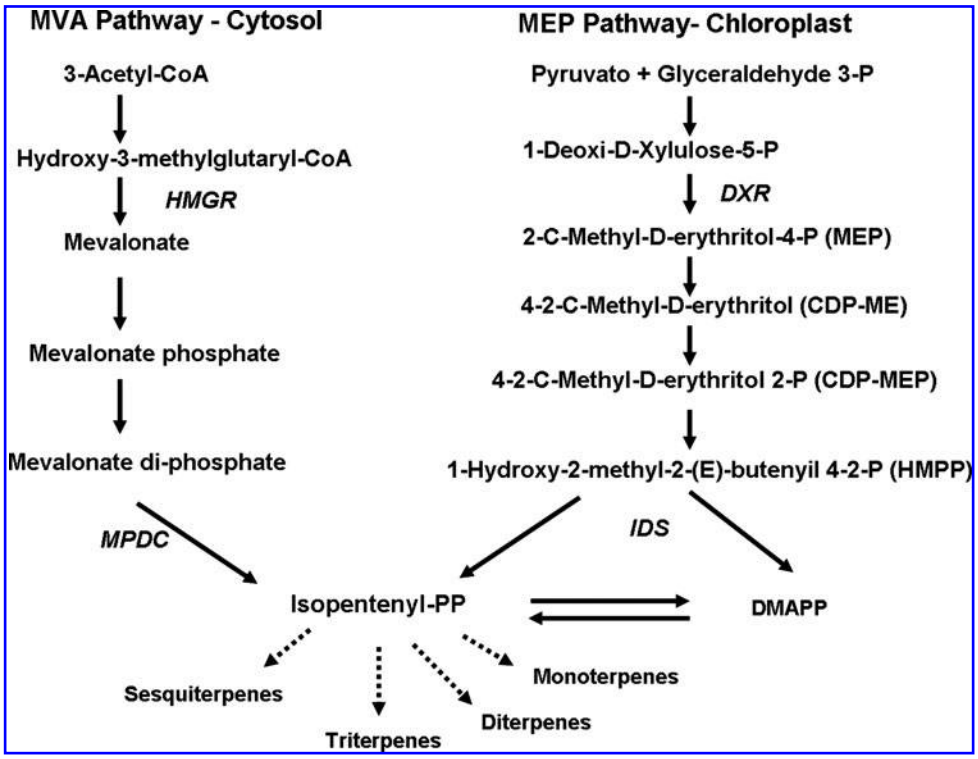

FIG. 1. Diagram of the isoprenoid pathway in the cytosol and in the plastids in plants.

methylglutaryl-CoA (HMG-CoA). This CoA derivative is reduced to mevalonate by HMG-CoA reductase (HMGR) (EC 1.1.1.34), the rate-limiting enzyme of the mevalonate pathway. Mevalonate is then phosphorylated twice and decarboxylated to form IPP, which is then converted to its isomer, DMAPP, by IPP isomerase. IPP and DMAPP synthesized in the mevalonate pathway are used as basic units in the biosynthesis of isoprenoids.

In higher plants, the gene *HMGR* is reported to be encoded by two or more isoforms. In *Arabidopis thaliana, Cucumis melo,* and *Lycopersicum esculentum HMGR* are encoded by two isoforms (Kato-Emori et al., 2001; Lange and Ghassemian, 2003; Narita and Gruissem, 1989), whereas in *Solanum tuberosum* and *Hevea brasiliensis* by three isoforms (Choi et al., 1992; Chye et al., 1992). These *HMGR* isoforms can be differentially expressed, depending on physiological conditions (Stermer et al., 1994; Weissenborn et al., 1995). Croteau et al. (2000) sugested that *HMGR* in plants is highly regulated and expressed in complex patterns, with individual genes exhibiting constitutive, tissue- or development-specific (Liao et al. 2009; Pateraki and Kanellis, 2010), hormones (Mansouri et al. 2009), or pathogen-inducible expression. An increase in *HMGR* transcription also has been reported after the application of salicylic acid or methyl jasmonate in *Salvia miltiorrhiza* and in xerophytes plants (Liao et al. 2009; Pateraki and Kanellis, 2010). Tissue wounding also stimulate an increase of HMGR transcription as well as other genes involved in the MVA and MEP pathway (Liu et al. 2010; Pateraki and Kanellis, 2010).

Herein, we report the characterization of a cDNA encoding a *C. arabica* HMG-CoA reductase. Also, the expression profile of two *CaHMGR* in various parts of the plant was investigated aiming to understand the process of formation of coffee isoprenoids.

Material and Methods

Plant materials

Fruits and tissues were harvested from 15-year-old plants of *Coffea arabica* cv. IAPAR 59 cultivated under field conditions at IAPAR (Instituto Agronômico do Paraná, Londrina, PR, Brazil). Fruits were collected every 4 weeks from flowering (September, 2005) up to complete maturation (May 2006). After collection, tissues were immediately frozen in liquid nitrogen and stored a −80°C before being analyzed. Fruit tissues (perisperm, endosperm, and pulp) were separated and used independently to extract total RNA.

In silico *analysis*

A keyword search for *HMGR* ESTs of *C. arabica* was carried out on the database of the Brazilian Coffee Genome Project (http:/www.lge.lbi.unicamp.br/cafe/). This database contained 130,792 expressed sequence tag (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 (Mondego et al., 2011). The sequences obtained were clusterized using the Sequencher v.4.1.4 software and BioEdit Sequence Alignment Editor v.7.0.8. For clusterization in the Sequencher the minimum match percentage and minimum overlap were 85 and 20%, respectively. Contigs were analyzed using the basic local alignment search tool (BLAST) in the tBLASTx mode at NCBI database.

Amplification of HMGRs

To check if the assembled reads were part of the same *HMGR* contig or formed a unique contig different primer combinations were designed for the purpose of amplifying

their open reading frames (ORFs) by RT-PCR. The primers designed according to conserved regions of related sequences were: (1) Fw-HMGRA 5′-GCC GGC GAA CAA CTC ATC AA-3′ in the sense orientation, (2) Rv-HMGRB 5′-TTA CGG CCT CCA TCA TGG TG-3′, and (3) Rv-HMGRC 5′- GCT GCA ATG GCT GAC ATC AGT-3′ in the antisense orientations. PCR reactions (25 ′L) were performed with 5 ng cDNA of fruit tissues, 10 mM TRIS-HCl pH8, 4; 50 mM KCL, 25 μM dNTP, 2.5 mM $MgCl_2$, 1.0 U de *Taq* polimerase (Invitrogen), and 10 pmol primer (sense and antisense). PCR conditions were: 2 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 1 min at 57°C, 2 min at 72°C, and a final cycle of 4 min at 72°C in a thermocycler PT-100™ (MJ Research, Waltham, MA, USA). PCR products were visualized in 1% agarose gels and stained with ethidium bromide. PCR products were eluted from the gel and purified by ethanol precipitation in presence of 10% (v/v) NaAc pH 5.2 and ressuspended in water, either for sequencing or for producing probes for hybridizations.

Sequencing and sequence analysis

Clones containing sequences of *CaHMGR1* were obtained from the clone collection of the Brazilian Coffee Genome Project. To obtain the complete sequence of *CaHMGR1* of coffee, the clone containing the cDNA insert with the 5′ end region of the contig HMGRA was resequenced using the primers Fw-HMGRA (5′-GCC GGC GAA CAA CTC ATC AA-3′) and Rv-HMGRB (5′-TTA CGG CCT CCA TCA TGG TG-3′). Sequencing was performed using DYEnamic™ ET Terminator Cycle Sequencing Kit (GE HealthCare, Piscataway, NY, USA) in a MegaBace 1000 sequencer. The sequence obtained was analyzed using the basic local alignment search tool (BLAST) in the tBLASTx mode at NCBI database. The consensus sequence was translated using the ORF Finder available at NCBI and aligned with homologous protein sequences. The SGN Intron Finder tool (Mueller et al., 2005) program was used for predicting the presence of exons/introns. The programs SignalP 3.0 and TMHMM2.0 (Emanuelsson et al., 2000) were used for predicting the presence of domains. Theoretical isoeletric point and molecular weight were calculated using ExPASy server (Gasteiger et al., 2003). The alignment with *HMGR* sequences of *L. esculentum*, *N. tabaco*, *C. arabica,* and *C. melo* was made using Vector NTI software (Invitrogen, Carlsbad, CA, USA), using standard parameters. Phylogenetic tree was constructed in Mega v.4.0 (Kumar et al., 2004) using UPGMA method. Bootstrap analysis was used to test the robustness of the UPMGA trees with 1000 replicates.

The nucleotide and deduced amino acid sequences of the full-length *CaHMGR1* cDNA and partial fragment of *CaHMGR2* gene described here have been submitted to GenBank and the assigned accession number are HQ540670 and HQ540671, respectively.

Southern blot analysis

For Southern blot analysis, 10 μg of DNA (Chang et al., 1993, with modification) of leaves of *Coffea arabica* cv. IAPAR 59, *C. eugenioides,* and *C. canephora* var. Apoatã were digested with restriction endonuclease *Dra*I, separated on a 0.8% (w/v) agarose gel, transferred to nylon membranes and hybridized with an internal fragment of 1,474 bp of *Coffea CaHMGR1* cDNA fragment labeled by random priming with 50 μCi of [α-^{32}P]dCTP according to Sambrook et al. (1989). Hybridization was performed overnight at 42°C using UltraHyb solution (Ambion, Austin, TX, USA) as previously described (Pereira et al., 2005). The membranes were washed as follows: two times SSC 1%; SDS 0.1% for 20 min and SSC 1%; SDS 0.1% for 20 min at 42°C. The images were captured using fluorescent image analyzer FLA 3000 series (Fuji Photo Film Co., Lts. Tokyo, Japan).

Analysis of expression by Northern blot

For analysis of expression by Northern blot 10 μg of total RNA was transferred to nylon membranes and hybridized with probes of *CaHMGR1* and *CaHMGR2* using UltraHyb solution as previously described for Southern blot. Total RNA was isolated (Chang et al., 1993, with modification) from different tissues of coffee like young flower buds (1.5 cm), mature flower (2.0 cm), expanded leaves (10 cm), young leaves (4.0 cm), branch, fruit, roots, pulp, perisperm, and endosperm from fruits of *C. arabica* cv. IAPAR 59 at different stages of maturation.

Results

In silico *analysis of* HMGR

A search for *HMGR* ESTs conducted on the database of the Brazilian Coffee Genome Project resulted in 13 ESTs from *C. arabica* that exhibited of 43–90% identity with HMGR gene of *Arabidopsis thaliana* (AY488113.1) at the nucleotide level. Assembly of all ESTs and alignment to *Arabidopsis HMGR* revealed three incomplete contigs that correspond to 5′ and 3′ regions of the *Arabidopsis* gene: contig A with 97–299 bp with 61% of identity (5′ end), contig B with 332–632 bp and contig C with 447–632 bp, with 83 and 89% of identity at the 3′ end (Fig. 2). The translated sequence of the contig A presented the highest similarity (73%) with the N-terminal region of a consensus *Nicotiana tabacum* (AAB87727) HMGR protein, but

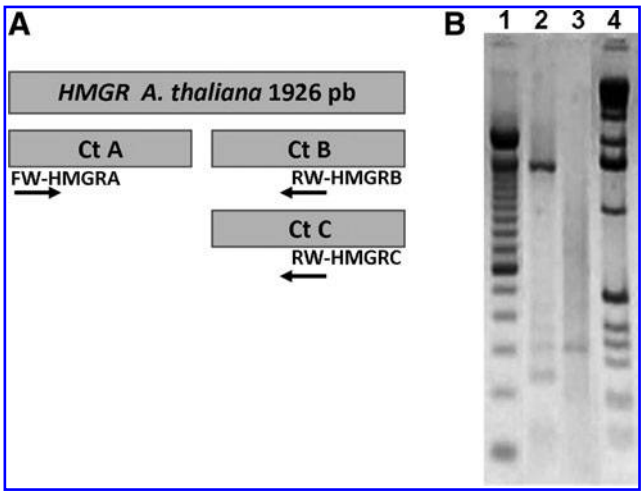

FIG. 2. Characterization of *HMGR* isoforms. (**A**) Contigs and their position in relation to a consensus *A. thaliana* sequence. (**B**) RT-PCR amplification of coffee *HMGR*. Columns: 1) Molecular weight 100 base pairs; 2) primers Fw-HMGRA and Rv-HMGRB; 3) Fw- HMGRA and Rv-HMGRC; 4) Molecular weight 1 kb.

with an incomplete C-terminal region. Contigs B and C also presented high similarity with the C-terminal region of the same protein, but they are incomplete at N-terminal region (Fig. 2A). The translated sequence of the contig C presented highest similarity (93% of identity) with the C-terminal region of a consensus *Morus alba* (AAD03789) HMGR protein. The alignment of nucleotide sequences of contigs B and C in NCBI database showed e-value $1e^{-75}$ and identities of 75% between them. These results suggested that there are at least two genes encoding the HMGR enzyme in *Coffea.* Using only the *in silico*

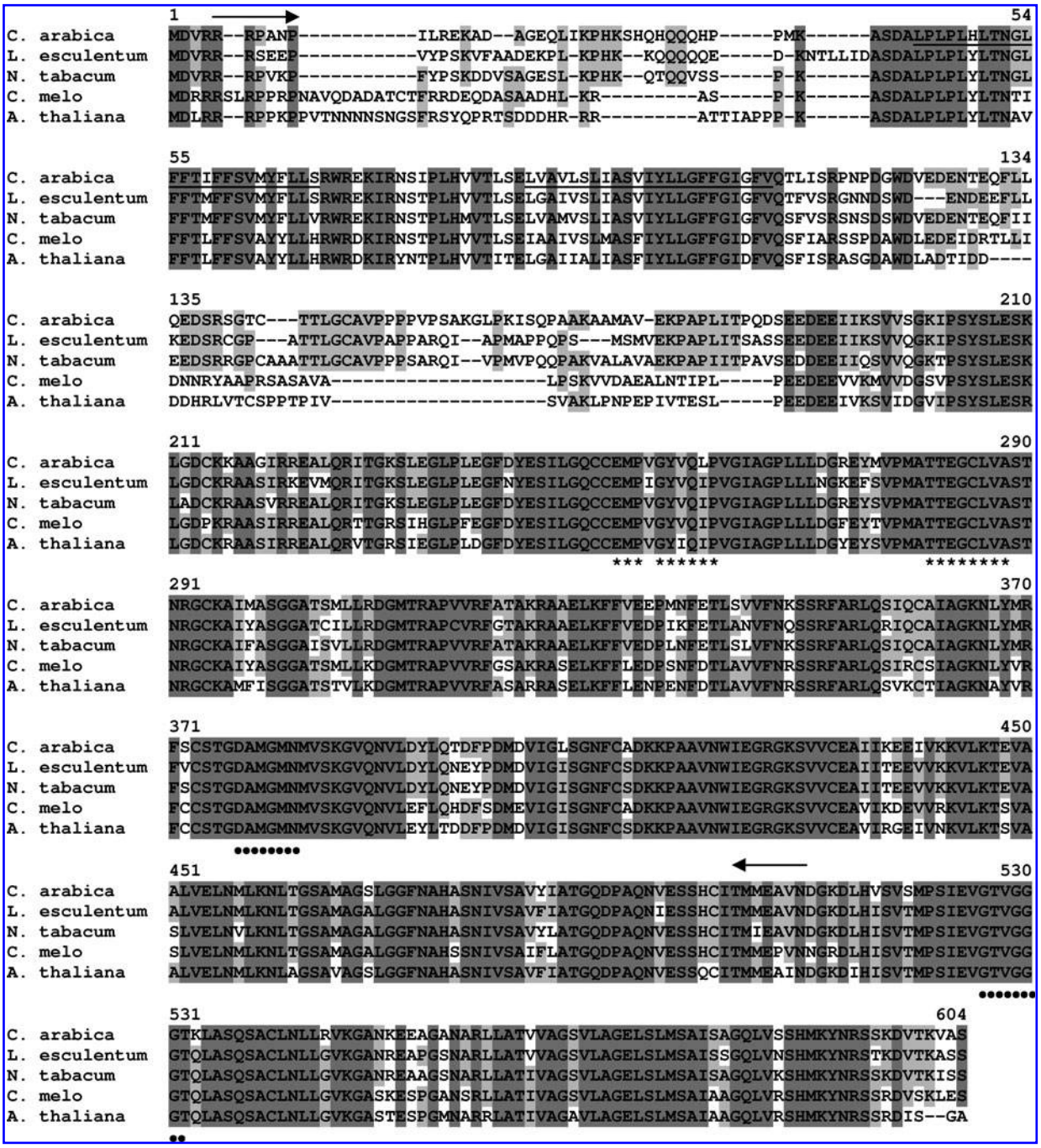

FIG. 3. Multiple alignment of the protein sequence deduced from the *Coffea arabica* with HMGR sequences from *Lycopersicon esculentum* (AAB62581), *Nicotiana tabacum* (AAB87727), *Cucumis melo* (BAA36291), and *Arabidopsis thaliana* (P14891). Identical amino acids are indicated with black foreground and thick gray background. Conserved amino acids are denoted with black foreground and light gray background. Two putative transmembrane regions are indicated underlined (LPLHLTNGLFFTIFFSVMYFLLS and LVAVLSLIASVIYLLGFFGIGFV). Two putative HMG-CoA binding sites (EMPIGYVQIP and TTEGCLVA) are indicated with asterisks and two NADP(H)-binding sites (DAMGMNM and GTVGGGT) are indicated with black dots. Arrows indicated the position of primers Fw-HMGRA and Rv-HMGRB used to amplify the cDNA fragment.

data, it was not possible to determine the complete cDNA sequence of *Coffea HMGR* isoforms.

Characterization and sequencing of a full-length HMGR *cDNA*

Primers were designed from highly conserved amino acid sequence of contigs A, B, and C to verify if the sequences of the three *HMGRs* contigs belong to the same or different isoform. When contig A forward primer (Fw-HMGRA) were used with the reverse primer of the contig B (Rv-HMGRB) for RT-PCR, it was possible to amplify a fragment about 1,560 bp, suggesting that they belong the same isoform, named *CaHGMR1* (Fig. 2B). No fragment was amplified using the combination forward primer of the contig A (Fw-HMGRA) and the reverse primer of the contig C (Rv-HMGRC), suggesting that the contig C is part of a different isoform, and thus called *CaHMGR2*. To close the gap between contigs A and B and obtain the complete sequence of Ca*HMGR1*, the clone forming contig A with the cDNA insert, which has the longest 5′-UTR sequence, was resequenced using the primers Fw-HMGRA and Rv-HMGRB. The cDNA sequence of the *CaHMGR1* comprises 2,242 bp, with the predicted presence of four exons and three introns (position 1186, 1368, 1715) with an ORF of 1,812 bp. The protein encoded by the *CaHMGR1* cDNA has 604 amino acid residues and a predicted molecular mass of 64.7 kDa and a deduced isoelectric point of 7.51.

The sequence analysis of the translated protein revealed that the functional motifs of *CaHMGR1* were very similar to those of other plant HMGRs with two HMG-CoA binding motifs (EMPVGYVQLP and TTEGCLVA) and two NADPH binding motifs (DAMGMNM and GTVGGGT). The Signal p1.1 program showed a prediction signal anchor for sequence of the *CaHMGR1.* Two transmembrane regions were identified by TMHMM2.0 analysis: one was located between the amino acids 46 and 68, and the other was located between amino acid 88 and 110 along the polypeptide chain (Fig. 3). The alignment of the sequence of *HMGR* from *C. arabica* with the sequences of several representatives *HMGRs* from other plants shows that more differences were found in the N-terminal region than in the C-terminal region (Fig. 3). The highest homology regions appeared around the substrate binding sites. Specifically, *CaHMGR1* exhibits 77% identity (84% similarity) to *HMGR2* of *Solanum tuberosu*m and 83% identity (89% similarity) to *HMGR* of *Nicotiana tabacum*, 79% identity (85% similarity) to *HMGR2 Lycopersicum esculentum* but only with 74% identity (85% similarity) and 68% identity (79% similarity) to *Arabidopsis thaliana HMGR1* and *HMGR2*, respectively.

Phylogenetic analysis of HMGR enzymes from different species of plants using a yeast gene as outgroup revealed two distinctive groups: one group formed by the two monocotyledonous species (*Oriza sativa* and *Zea mays*), and other by dicots (Fig. 4). The deduced amino acid sequence of *CaHMGR1* formed a clad with sequences from the Solanacea family.

Southern blot analysis

Southern Blot analysis using a C*aHMGR1* probe was conducted to analyze the structure of the gene in *Coffee spp.* For the hybridization, the ORF region derived from cDNA was used as probe and the hybridization was carried out with high stringency washing. The hybridization showed a similar pattern of bands in *C. eugenioides* and *C. canephora* (three

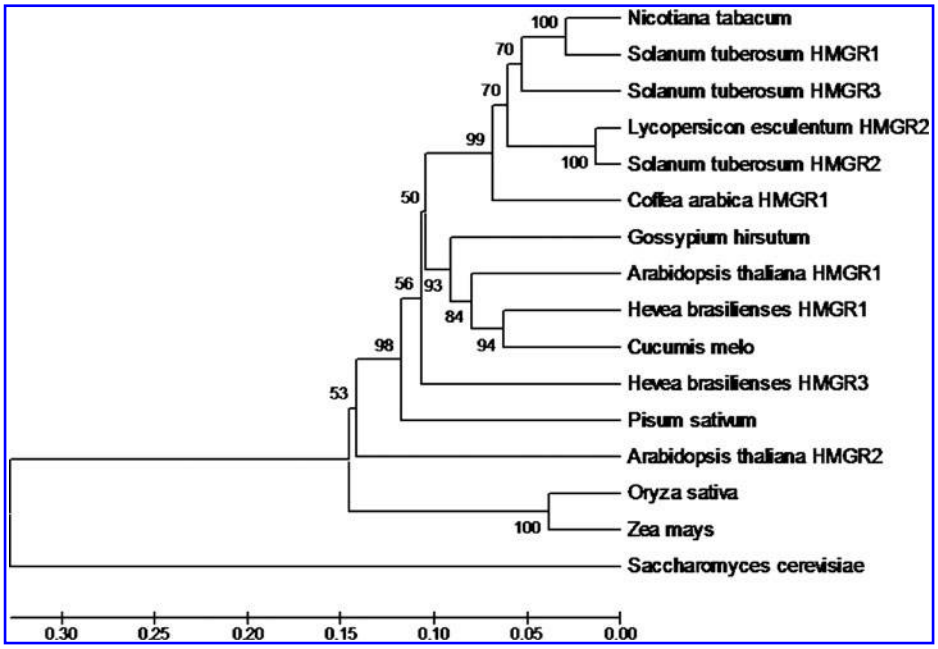

FIG. 4. Unrooted dendogram of *HMGR* sequences constructed by UPMGA method using Mega program. The sequences had been obtained through NCBI (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/): *Nicotiana tabacum* AAB87727; *Solanum tuberosum* HMGR1, P48020; *Solanum tuberosum* HMGR3, U51986; *Lycopersicon esculentum* AAB62581; *Solanum tuberosum* HMGR2, U51985; *Gossypium hirsutum* AAC05089; *Arabidopsis thaliana* HMGR1 P14891; *Hevea brasiliensis* HMGR1, X54659; *Cucumis melo* BAA36291; *Hevea brasiliensis* HMGR3, Q00583; *Pisum sativum* AAL37041; *Arabidopsis thaliana*HMGR2, L19262; *Oryza sativum* Q9XHL5; *Zea mays* O24594; *Saccharomyces cerevisiae* EDN64320.

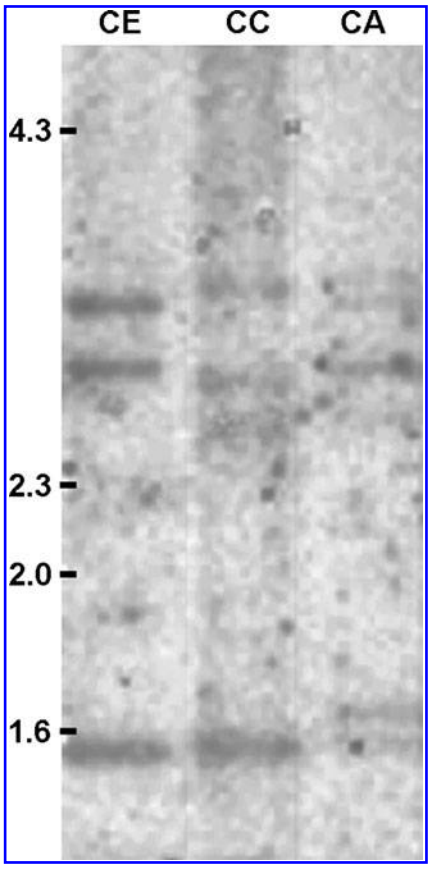

FIG. 5. Southern blot analysis of *Coffea eugenioides* (CE), *Coffea canephora* (CC), *Coffea ara*bica (CA), genomic DNA hybridized with *CaHMGR1* probes. Genomic DNA digested with *Dra*I restriction enzymes were fractionated by electrophoresis on 0.8% (w/v) agarose gel, and transferred to a nylon (Hybond-N^+) membrane. Hybridizations were carried out with the probe *CaHMGR1* corresponding to a 1,474 pb internal fragment of CaHMGR1 cDNA. Molecular weight standards are indicated at the left in kilobases.

bands) (Fig. 5). In *C. arabica* a similar pattern was observed but with two more bands, probably due the tetraploid characteristic. These results suggest that *CaHMGR1* belongs to a family of *HMGR* genes, with two or three copies for *C. eugenioides* and *C. canephora* and five or three copies for *C. arabica*. The probe *CaHMGR1* did not show cross hybridization with *CaHMGR2* probe (data not shown).

Transcription levels of HMGRs

Northern blots analysis with the *CaHMGR1* probe detected transcripts in young flower buds, flower buds, and young leaves. It was very weak in expanded leaves and fruits, and it was not detected in roots and branches (Fig. 6). Cafestol and kahweol, originated from the isoprenoid pathway, are produced mainly in coffee fruits tissues. Transcritps of *CaHMGR1* probe were verified in pulp, perisperm, and endosperm of coffee fruits during maturation. Although a weak detection of transcripts in whole fruits was observed, Northern blots analysis with the *CaHMGR1* probe revealed transcripts during all maturation stages in pulp [55–251 days after flowering (DAF)], at the initial stage of development of perisperm (55

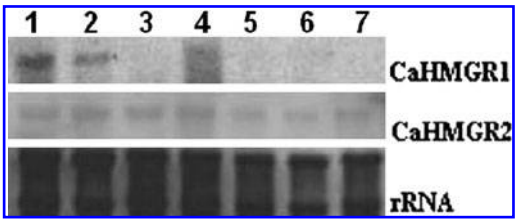

FIG. 6. Transcription pattern of *CaHMGR1* and *CaHMGR2*. Total RNA (15 μg) isolated from of (1) young flower buds; (2) mature flower buds; (3) expanded leaves; (4) young leaves; (5) branch; (6) fruit and (7) root of *C. arabica*. IAPAR 59 was separated in a formaldehyde-agarose gel and transferred onto a nylon membrane. Membranes hybridized with probes marked with dCTP α-32P. Total RNA: gel of the samples for comparison of the used concentration.

DAF) and very weak signals in endosperm (83 DAF) (Fig. 7). Meanwhile, *CaHMRG2* transcripts were observed in all samples analysed, in all different tissues of coffee fruits during maturation, as well as flower buds, leaves, branch, and roots (Figs. 6 and 7).

Discussion

Here we report that, through the application of bioinformatics analysis to the Coffee Genome Project EST database, we obtained the sequences of two 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) isoforms. In addition, we identified a full-length clone encoding this enzyme and we reported the expression data in various coffee tissues. In *Arabidopis thaliana, Cucumis melo,* and *Lycopersicum esculentum HMGR* are encoded by two isoforms (Kato-Emori et al., 2001; Lange and Ghassemian, 2003; Narita and Gruissem, 1989), in *Solanum tuberosum* and *Hevea brasiliensis* by three isoforms (Choi et al., 1992; Chye et al., 1992). The *in silico* analysis based on ESTs data associated with the PCR results indicated the expression of only two isoforms of *HMGR* in *Coffea*. Although it is posssible that another isoform is transcribed, the number of ESTs analysed (more than 153,000 for *C. arabica* plus 47,000 for *C. canephora*) strongly suggests that only two isoforms of *HMGR* may have an important role for isoprenoid biosynthesis via the mevalonate pathway in *C. arabica.* Wu et al. (2009), also identified only two *HMGR* isoforms after analyzing 6678 ESTs from flower, leaf, and root cDNA of American ginseng (*Panax quinquefolius* L.).

According to the *in silico* analyses, the deduced *CaHMGR1* of coffee showed considerable identities with other known plant HMGRs proteins and contained two transmembrane domains, two HMGR binding motifs, and two NADPH binding motifs (Fig. 3) as in *Cucumis melo* (Kato-emori et al., 2001), *Taxus media* (Liao et al., 2004), and *Corylus avellana* (Wang et al., 2007). The protein encoded by the *CaHMGR1* cDNA has a predicted molecular mass and a deduced isoelectric point that is very similar to the previously reported for other plants (Liao et al., 2004; Shen et al., 2006; Wang et al., 2007). The strongest amino acid homology was found to HMGR enzymes of *N. tabacum, S. tuberosum,* and *L. esculentum.* Phylogenetic relatedness of *CaHMGR1* to known HMGRs from the Solanaceae family is illustrated in Figure 4. The tree shows the early split of monocots and dicots with a separate branch for yeast. The clad

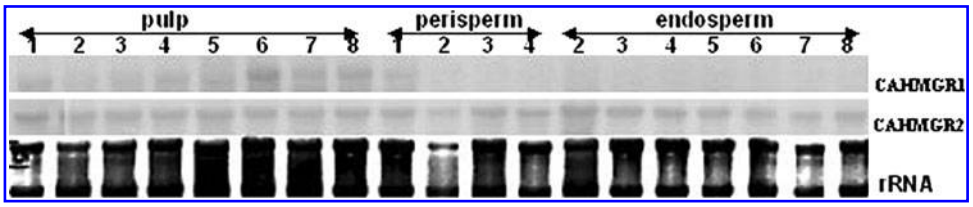

FIG. 7. Transcription pattern of *CaHMGR1* and *CAHMGR2*. Total RNA (15 μg) isolated from pulp; perisperm and endosperm of fruits of *C. Arabica* cv. IAPAR 59. Columns 1 to 11 correspond to months after flowering (AF) from the period of October (30 days AF) to May (240 days AF). Membranes hybridized with probes marked with dCTP $^{\alpha}P^{32}$. Total RNA: gel of the samples for comparison of the used concentration.

formed with *CaHMGR1* and HMGR sequences of especies from the Solanaceae family corroborate with reports showing the proximity between *Coffea* from the Rubiaceae family with the Solanacea (Lin et al., 2005).

To investigate whether the *CaHMGR1* gene belongs to a multigene family, genomic DNA of *C. arabica* and its parents, *C. canephora* and *C. eugenioides* were digested with *Dra*I that had no restriction site within the probe region. For all three species, there were more than two distinct bands ranging from 4.3 kb to less than 1.6 kb (Fig. 4), which might be speculated that more than one sequence with homology to *CaHMGR1* gene were present in *Coffea* genome, although there is also the possibility for the presence of restriction sites in the intron sequence of *CaHMGR1*. Venkatachalam et al. (2009) reported the presence of three introns in a rubber three *HMGR* gene. It was suggested that more than two copies of *HMGR* gene may exist in *Solanum tuberosum* and *Hevea brasiliensis* (Choi et al., 1992; Chye et al., 1992; Venkatachalam et al., 2009). So the possibility cannot be excluded that *CaHMGR1* might be a two-copy gene, and the number of *CaHMGR1* genes from *C. arabica* will be elucidated when more genomic information is available.

Low abundant transcripts of both *CaHMGR1* and *CaHMGR2* were detected by Northern blots. An additional confirmation of low *HMGR* transcription is that the sequence of the two isoforms of this gene were found only 13 times in a search of more than 200,000 ESTs from 37 cDNA libraries constructed by using mRNAs isolated from different tissues at different developmental stages. Analysis of coffee *HMGR* genes expression in all fruit tissues during the developmental stages studied revealed that the *CaHMGR2* gene was expressed constitutively in all tissues examined but at very low levels (transcripts became visible only after 18 h exposure to the fluorescent image analyzer) (Figs. 6 and 7). Similar results were observed by Dudareva et al. (2005), who reported a constitutive and low gene expression level of a *HMGR* gene in leaves and floral tissues of snapdragon flowers (*Antirrhinum majus)*. On the other hand, Northern blot results indicated abundant mRNAs encoding *CaHMGR1* was only transcribed in floral buds and young leaves, whereas a weak signal was detected in whole fruits of *C. arabica.* The isoprenoids cafestol and kahweol are produced mainly in fruits of *C. arabica* (Speer and Kolling-Speer, 2001); however, fruits analysed in Northern blot are constituted of pulp and mainly endosperm (Geromel et al., 2006), which explain the weak detection of transcripts in whole fruits. Northern blot from developmental stages of fruit tissues detected *CaHMGR1* transcripts in pulp, mainly at the initial stages of perisperm and in the endosperm. The transcription of *CaHMGR1* for the perisperm tissue correlates to the observed amount of diterpenes kahweol and cafestol in this tissue reported by Dias et al. (2010), but surprisingly, did not show the amount of transcription expected for the endosperm. The overall low transcription level of both *HMGR* isoforms in coffee suggests that the activity of this enzyme may be subject to both posttranscriptional and posttranslational regulation. Dudareva et al. (2005) showed that *HMGR* is expressed constitutively in all tissues of *Antirrhinum majus* but at very low levels. Laule et al. (2003) and Nieto et al. (2009) showed the lack of correlation between gene expression patterns and the accumulation of isoprenoid metabolites in *Arabidopsis*, indicating that posttranslational processes may play an important role in regulating flux through isoprenoid metabolic pathways. The *HMGR* gene belongs to the MVA pathway, which is localized at the cytosol in opposition to the plastidic MEP pathway. Although the two pathways to isoprenoids (MAV and MEP) are compartmentalized, there is evidence that they cooperate in the formation of certain isoprenoids, probably as a consequence of metabolic crosstalk between the pathways via the plastid envelope membrane (Bick and Lange, 2003). These authors suggest the presence of an unidirectional proton symport system in plastid membranes for the export of specific isoprenoid intermediates between cytosolic and plastidial pathways. As the capacity of the MEP pathway to function or compensate the inhibition of MVA pathway is more pronounced than the opposite (Laule et al., 2003), it is tempting to speculate that the low gene expression level in coffee of both HMGRs, the key regulatory enzyme of the MVA pathway, indicates that the cytosolic pathway may not be the principal responsible for the production of isoprenoid precursors in coffee plants. Further experiments using RTqPCR for analysis of the transcription pattern of *CaHMGR1* and *CaHMGR2* from the MVA pathway, and *DXR* for the MEP pathway are being conducted in our lab.

To our knowledge, *CaHMGR1* is the first reported gene involved in the MVA pathway from *C. arabica*. Further characterization of *CaHMGR1* gene expression as well as other genes involved in the MVA and MEP pathways it will be very helpful to understand the importance of the cytosolic and plastidic pathways involved in the isoprenoid metabolism and their contribution for the terpenes biosynthesis. Furthermore, those genes can be potential targets for bioengineering terpenes in coffee beans.

Conclusions

The *in silico* and molecular analysis indicated the presence of only two isoforms of HMGR in *Coffea arabica*. For the

deduced amino acid sequence of *CaHMGR1*, the strongest homology was found to HMGR enzymes of *N. tabacum*, *S. tuberosum,* and *L. esculentum*. Analysis of coffee HMGR genes expression revealed that the *CaHMGR2* gene was expressed constitutively in all coffee tissues but at very low levels and *CaHMGR1* was transcribed in floral buds, young leaves, in pulp. Transcription was detected in whole fruits and mainly at the initial stages of perisperm but at low levels in endosperm.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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