

In Silico and in Vitro Analysis of the Isoprenoid Pathway in Coffee

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SUMMARY

The most important lipids in coffee, the diterpenes khaweo and cafestol, are originated from the isoprenoid pathway. 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). In higher plants, there are two independent pathways located in the cytosol (mevalonic acid or MVA pathway) and in the plastids (methylerythritol phosphate – MEP – or non mevalonic pathway). Throughout the data mining of the Brazilian Coffee Genome Project we studied the genes that code for the enzymes 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and mevalonate diphosphate decarboxylase (MPDC) for the MVA pathway and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and isopentenyl diphosphate/dimethylallyl diphosphate synthase (IDS) for the MEP pathway.

INTRODUCTION

Coffee is one of the most important world agricultural commodities. Although the cup quality is one of the most important aspects for consumption, very little is known about several components, which can contribute for quality, including lipids. The most important lipids in coffee, the diterpenes khaweo ($C_{20}H_{26}O_3$), and cafestol ($C_{20}H_{28}O_3$), are originated from the isoprenoid pathway. 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) (Rodriguez and Boronat, 2002). In higher plants, two independent pathways located in separate intracellular compartments are involved in the biosynthesis of IPP and DMAPP (Figure 1). In the cytosol, IPP is derived from the mevalonic acid (MVA) pathway (Qureshi and Porter, 1981; Newman and Chappell, 1999), that starts from the condensation of acetyl-CoA, whereas in plastids, IPP is formed from pyruvate and glyceraldehyde 3-phosphate through the methylerythritol phosphate (MEP or non-mevalonate) pathway (Esenreich et al., 1998; Rohmer, 1999). The key enzyme of the cytoplasmic MVA pathway is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) (EC 1.1.1.34). *HMGR* in plants is reported to be encoded by two genes (Bach et al., 1991; Weissenborn et al., 1995). These *HMGR* isoforms are differentially expressed, depending on physiological conditions (Weissenborn et al., 1995; Stermer et al., 1994). The enzyme mevalonate diphosphate decarboxylase (MPDC) is responsible for the formation of IPP.

For the MEP pathway, the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is currently considered as the first specific step for biosynthesis of isoprenoid in plastids. The last enzyme on the MEP pathway is the isopentenyl diphosphate/dimethylallyl diphosphate synthase (IDS), which can form either IPP or DMAPP. With the aim to understand the process of formation of the isoprenoids in *Coffea* we have started the characterization of the genes involved on the MVA and MEP pathway.

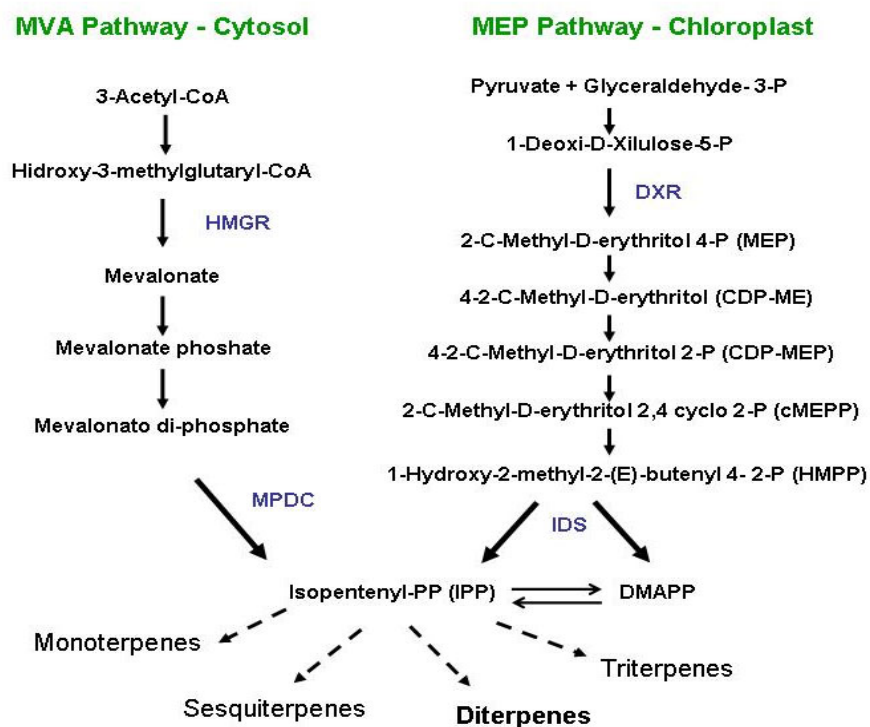


Figure 1. Diagram of the isoprenoid pathway in the cytosol and in the plastids in plants.

MATERIAL AND METHODS

A key word search for ESTs from genes of the MAV and MEP pathway was conducted on the database of the Brazilian Coffee Genome Project (Vieira et al., 2006) (<http://www.lge.lbi.unicamp.br/cafe/>). The sequences were clusterized for contig formation using the Sequencher 4.5 software. Singlets and contigs were analyzed using the basic local alignment search tool (BLAST) in the tblastx mode at NCBI database. The consensus contig were translated using the Open Reading Frame Finder available at NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The proteins sequences were aligned with homologous sequences from other organisms using Clustal W at <http://clustalw.genome.ad.jp/>.

Total RNA was isolated from pulp, perisperm and endosperm from fruits of *Coffea arabica* cv. IAPAR 59 at different stages of maturation (Chang et al., 1993). One µg of total RNA was used to produce cDNA with ThermoscriptTM oligo DT System (Invitrogen). To check the complementation of the HMGR contigs, different primer combinations were used on PCR reaction of the cDNA. For Northern Blot analysis 10 ug of total RNA was transferred to nylon membranes and hybridized using UltraHyb solution as previously described (Pereira et al., 2005).

RESULTS AND DISCUSSION

The keyword search for the *DXR* (MEP pathway) resulted in twenty two ESTs and formed only one full length contig. Electronic Northern showed higher expression in libraries of germinating seeds (Table 1). Search for *IDS* ESTs retrieve 47 sequences, which also formed only one full length contig. The sequences were originated mainly from hypocotyls induced with acylbenzolar-S-methyl, a SAR inducer. It is interesting to observe that several of ESTs coming for the MEP pathway were obtained from cell tissue culture libraries or stress induced libraries. On the other hand, the number of ESTs from the two genes from the MVA pathway (HMGR and MPDC) on those libraries was very low (Table 1).

Table 1. Eletronic Northern of genes from the MVA and MEP pathway.

Gene	ESTs	Fruits and flowers buds	Leaf	Tissue culture cells	Walter stress	Hypocotyl/ acilbenzolar	Germinating seeds
CAHMGR1	11	8	2	1			
CAHMGR2	2			2			
MPDC	7	2		4		1	
DXR	22	4	3	6	3	1	5
IDS	47	9	9	14	4	10	1

For the MVA pathway, 13 ESTs were found for HMGR that originated three incomplete contigs named: 6479, 6175 and 0003. For MPDC only 7 ESTs were found forming one full lenght contig.

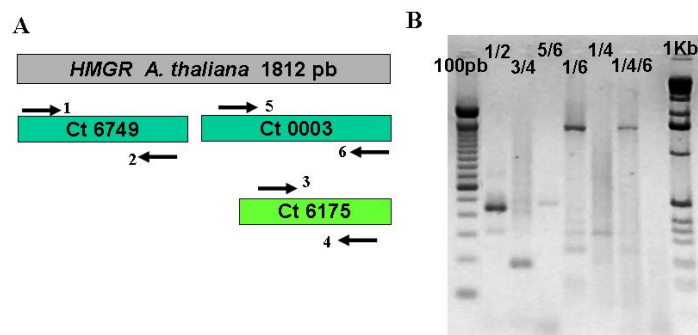


Figure 2. Characterization of *HMGR* isoforms by PCR. A) Contigs obtained and their position related to a consensus *A. thaliana* sequence. Number on the arrows indicated the primer position for fragment amplification. B) Partial PCR amplification of coffee *HMGR*. Numbers in the columns indicated the used primer combination. Expected sized for the fragments: 1/2: 6749/6749C = 450 pb; 3/4: HMGR 0/0C = 186 pb; 5/6: HMGR 6175/6175C = 483 pb; 1/4: 6749/0C= 1556pb; 1/6: 6749/6175C=1694pb; 1/4/6: 6749/0C/6175C = 1556pb.

For HGMR, the translated sequence of the contig 6749 presented high similarity with the N-terminal region of a consensus *Arabidopsis thaliana* HMGR protein. Contigs 6175 and 0003 presented high similarity with the C-terminal region of the same protein (Figure 2A) As the presence of two isoforms were expected, primers based on those contigs were used to check whether they could belong to the same contig 6749 or represent another *HMGR* isoform. When we used primers from sequences of contig 6749 and 0003, it was possible to amplify a fragment corresponding to the expected size of the full-length cDNA, indicating that they are

the same isoform (Figure 2B, primers 1 and 6), and was called *CaHGMR1*. The combination of primer 6749 and 6175 did not amplify the expected fragment size, suggesting that 6175 is a different isoform, called *CaHMGR2*.

The number of isoforms of the four genes datamined was the same reported for *Arabidopsis thaliana*, with the exception for the *MPDC* that has two isoforms in *A. thaliana* and only one in the Brazilian Coffee Genome Project *C. arabica* ESTs database. A keyword search from *MPDC* in *C. canephora* ESTs (LLIn et al., 2005) using the Harvest platform (<http://harvest.ucr.edu/>) also retrieved only one contig that was mainly expressed during the late stages of fruit maturation.

REFERENCES

- Bach, T. J.; Wettstoln, A.; Boronat, A.; Forro, A.; Enjuto, M.; Grulssom, W.; Narita, J. O. In Physiology and Biochemistry of Sterols. (PATTERSON, G. W.; and NOS, D. W., ed) American Oil Chemists Society, p. 29-49, 1991.
- Chang, S.; Puryear J.; Cairney, J. A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol*, 11:113-116, 1993.
- Esenreich, W.; Schwarz, M.; Cartayrade, A.; Arigoni, D.; Zenk, M. H.; Backer, A. The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. *Chemistry Biology* 5: 221-233, 1998.
- Lin, C.; Mueller, L.A.; Mc Carthy, J.; Crouzillat, D.; Pétiard, V.; Tanksley, S.D. Coffee and tomato share common gene repertoires as revealed by deep sequencing of seed and cherry transcripts. *Theor Appl Genet.* 112(1):114-30, 2005
- Newman, J. D.; Chappell, Isoprenoid Biosynthesis in Plants: Carbon Partitioning Within the Cytoplasmic Pathway. *Crit. Rev. Biochemistry Mol Biol* 34: 95-106, 1999.
- Pereira, L. F. P.; Galvão, R. M.; Kobayashi, A. K.; Cação, S. M. B.; Vieira, L. G. E. Ethylene production and acc oxidase gene expression during fruit ripening of *Coffea arabica* L. *Braz. J. Plant Physiol.* 17(3): 283-289, 2005.
- Qureshi, N.; Porter, W. In Biosynthesis of Isoprenoids Compounds, eds. PORTER, J. W. & Spurgeon, S. L. (WILEY, New York), p. 47-94, 1981.
- Rodriguez M.; Boronat, A. Elucidation of the Methylerythritol Phosphate pathway for Isoprenoid Biosynthesis in Bacteria and Plastids. A Metabolic Milestone Achieved through Genomics. *Plant Physiology*, vol. 130:1079-1089, 2002.
- Rohmer, M. In Comprehensive Natural Product Chemistry, ed. Cane, D. E. (Pergamon, Oxford), 45-68, 1999.
- Stermer, B. A.; Biancehini, G. M.; Korth, K. Regulation of HMG-CoA reductase activity in plants. *J. Lipid Res.* 35:1133-1140, 1994.
- Vieira, L.G.E.; Andrade, A.C.; Colombo, C.A.; et al. Brazilian coffee genome project: an EST-based genomic resource. *Braz. J. Plant Physiol.* 18(1):95-108, 2006
- Weissenborn D. L.; Denbow C. J.; Laine M.; Lang S. S.; Zhenbiao Yang ; Xueshu YU ; Cramer C. L.; HMG-CoA reductase and terpenoid phytoalexins: molecular specialization within a complex pathway *Physiol. Plantarum*: 1995, vol. 93, n°2, pp. 393-400

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