Transcriptional Profile of Genes Involved in the Biosynthesis of Phytate and Ferritin in *Coffea*

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The present work aimed to study the control of the biosynthesis of the antinutritional factor phytate and its associated Fe-rich protein family, ferritin, in coffee. Phytate has the ability to chelate Fe, making it unavailable to human absorption. The *Coffea* genome databases were queried for genes associated with phytate metabolism and ferritin genes. The genetic framework for phytate biosynthesis and its reverse pathway was identified in silico analyses and indicate that *Coffea* phosphatidyl inositol kinase and monophosphatase families play nonredundant roles in phytate metabolism. The transcriptional profiles of phytate biosynthesis key-genes *MYO-INOSITOL(3)P1 SYNTHASE*, two genes coding for PHOSPHATIDYL INOSITOL KINASE, and three *FERRITIN* genes were temporally evaluated by qPCR in coffee seeds from two crop locations, Adamantina-SP and Ouro-Fino-MG, the last one traditionally associated with high-quality coffee beverage grain. A targeted metabolome profile of phytic acid contents throughout three fruit maturation stages in association with the transcriptional analysis was also obtained. Taken together, our data indicate that the investigated local conditions did not cause significant alterations in phytate biosynthesis. Futhermore, the temporal transcriptional profiling revealed that candidate gene expression is regulated independently of phytate accumulation. In contrast, the expression profile of ferritin-unit genes is affected by environmental conditions and genetic background. The roles of the investigated genes are discussed concerning the quality of coffee beverage.

KEYWORDS: Antinutritional factor; ferritin; iron; myo-inositol; phytate; qPCR

INTRODUCTION

Coffee is among the five most valuable agricultural exports from developing nations and ranks second in Brazilian international trade exchanges, which represents more than one-third of the global coffee production and exports. The crop's economical importance is reflected in the financial support provided to the recent Brazilian Coffee Genome Project (BCGP), in which more than 200.000 ESTs from 37 cDNA libraries of *Coffea arabica*, *C. canephora*, and *C. racemosa* were sequenced, yielding a total of 33,000 distinct unigenes (*1*). The project (http://www.lge.ibi.unicamp.br/cafe) aimed to develop advanced molecular biology tools to coffee breeding and physiology study programs.

Quality is one of the most important traits considered in selection criteria for coffee improvement, according to requirements from several levels (e.g., farmers, exporters or importers, rosters, and consumers of coffee beverages) (*2*). The quality attribute has been traditionally associated with the environmental conditions of the cultivation areas. More recently, bioactive compounds and nutraceutical properties have also been considered as quality components of human and animal foods and beverages (*3*, *4*). Despite its importance, the physiological, genetic and molecular bases for nutritional proprieties in coffee cherries and beverages remain elusive.

Iron is one of the most important micronutrient beneficial for human health and low levels of the mineral are frequently associated with anemia. In plant breeding programs, two main strategies are promising to enhance iron contents and availability in seeds of crop plants; increase ferritin expression (*5*) and induce the generation of low phytic acid (lpa) seeds (*6*). Ferritin, a class of Fe storage protein well-conserved throughout plant evolution, is encoded by a four-gene family in the *Arabidopsis thaliana* genome (*7*). Phytate (Ins P6), *myo*-inositol 1,2,3,4,5,6-hexakisphosphate, is a major P storage compound in plant seeds, commonly found as deposits of mixed phytate salts of mineral cations K, Mg, Ca, Mn, and Zn (*8*). High phytate contents in grains induce chelating effects upon metal ions, such as Fe and Zn. Therefore, there is a close metabolic link between nutritional Fe and *myo*-inositol metabolism. Fe sequestration by phytate chelates severely reduces its availability to animals, leading to several degrees of malnutrition. In agriculturally important crops, low phytic acid contents have been investigated in mutants, such

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as in maize (*9*) and soybean (*10*), or by employing transgenic approaches (*11*, *12*).

Because of its chemical composition, consisting mainly of antioxidants, coffee has been suggested to be a functional beverage (*13*). However, coffee is not a good source of iron (*14*), and it has been demonstrated that coffee drinking interferes with intestinal Fe absorption in humans (*15*). This detrimental effect seems to be associated with coffee polyphenols, which are thought to bind nonheme iron (*16*). The role of phytic acid as an iron chelator remains elusive in coffee.

The main goal of the present work was to investigate the genetic, genomic, and physiological aspects of phytic acid metabolism and the ferritin family in *Coffea*, providing tools for plant breeding programs to access complex traits as the properties of coffee beverage. Initially, the metabolic aspects of interest were investigated by in silico analyses that provided the genetic framework for phytate metabolism and the ferritin gene family in *Coffea*. Subsequently, genomic and biochemical tools were used to evaluate the relationship between the transcriptional profile of candidate genes and phytic acid components in coffee seeds grown in two locations; one of which is traditionally associated with high-quality coffee. Four fruit maturation stages were investigated for the evolution kinetics of gene expression and phytate components.

MATERIAL AND METHODS

In Silico Analyses. *Database Searches and Alignments.* Homologues of *A. thaliana* components of phytate metabolism and the ferritin family were identified in BLAST searches (*17*) against Coffee Genome Project databases, constituted of approximately 200,000 ESTs obtained from sequencing of 37 libraries (*1*). Data validation was performed by tBLASTx and tBLASTn searches of the retrieved sequences against the locally built GenBank database. Sequences failing to retrieve the original sequence used to query the database were eliminated from the projects. The resulting alignments were filtered by a threshold e-value of 1e-15, and the hits were further analyzed for functional domain description. Validated sequences were translated, and protein (deduced amino acid) alignments were performed using ClustalX (*18*).

Motif Analysis and in Silico Characterization. The identified orthologues were further investigated for the presence and sequence conservation of recognizable functional domains described in several protein analysis and gene function databases (EMBL-EBI, www.ebi.ac.uk/interpro/; ExPaSy from SIB, http://www.expasy.org/prosite/; GO, http://www.godatabase.org/cgi-bin/amigo/go.cgi; Pfam, http://www.sanger.ac.uk/Software/Pfam/).

In Silico Gene Expression Analysis. Qualitative gene expression profiling was performed by in silico analyses using virtual Northern blots. The gene of interest was used in queries against reference-sequence databases, generating an alignment of the input gene to its paralogues. The resulting alignment was used to find sequences in the entire mRNA input that are specific to the gene (probe). The resulting alignments were collectively used to query the EST database again using BLAST. This heuristic attempts to avoid false-positives or ESTs from a paralogue of the input gene rather than the gene itself. The frequency of reads of each EST contig in a given library was calculated and normalized according to the total number of reads from the investigated library and the total number of reads in all libraries. A correlation matrix between EST contigs and libraries was then generated, and gene expression patterns among ESTs and libraries were obtained by hierarchical clustering based on the Spearman Rank correlation matrix using Cluster v.2.11 software ((*19*) http://rana.lbl.gov/EisenSoftware.htm), by substituting the clusters by their average expression pattern. Graphic outputs were generated using Tree View v.1.6 software (http://rana.lbl.gov/EisenSoftware.htm) and presented in grayscale.

Plant Material. Fruits from two *C. arabica* cultivars (Obatã and Catuaí Amarelo) were harvest in two distinct locations, growing at field conditions; Adamantina, State of São Paulo, Brazil (21°40′60″S, 51°4′0″W, 437 m) and Ouro Fino, State of Minas Gerais, Brazil (20°23′08″S, 43°30′29″W, 1179 m). Annual highest and lowest mean temperatures in Adamantina were 29.7 and 17.6 °C, respectively, and 24.0 and 14.4 °C in Ouro Fino. Micronutrient soil analysis for the locations exhibited no deficiency according to the recommendation by Raij et al. (*20*). Fe soil content was 54 mg/dm^3 for the Catuaí Amarelo plot and 30.5 mg/dm^3 for the Obatã plot in Ouro Preto, and 15.5 mg/dm^3 for the soil in Adamantina, where both cultivars were grown together. Traditionally, the quality of the coffee from Ouro Fino is higher than that from Adamantina. Cherries were harvested from January to September (Ouro Fino) and January to May (Adamantina) in 2007, at the following stages of fruit development: 1, green stage with liquid endosperm (G1); 2, green stage with endosperm at milky stage (G2); first cherry stage with the remaining outer layer perisperm green (CR1); second cherry stage with the remaining outer layer perisperm silver or silver skin, pericarp maturation (CR2) (*21*). In order to evaluate the biological replicates, fruits from both genotypes grown in both locations were separately harvested from three trees located at the same plot. Cherries were dissected to separate the endosperm from the other fruit tissues (pericarp and perisperm), and the endosperm was used for RNA extraction.

RNA Extraction, cDNA Synthesis, and Quantitative PCR. Total RNA was isolated using the TRIZOL reagent (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. RNA quality was assessed by electrophoresis on agarose 1% (w/v) gels stained with GelRed (Biotuim, Inc., Hayward, California, USA) and visualized using a fluorescent image analyzer FLA-3000 (Fujifilm Corporation, Tokyo, Japan). RNA quantification was performed by spectrometry at 260−280 nm. Total RNA (1.5 μg) was treated with 2 units of DNase I (Promega Corporation, Madison, Wisconsin, USA) and used in the synthesis of the cDNA, using the kit ImProm II Reverse Transcription System (Promega Corporation, Madison, Wisconsin, USA). Quantitative PCR (qPCR) was carried out using Applied Biosystems 7500 Fast Real-Time PCR System and a *SYBR Green*-based detection system (*SYBR Green PCR Master Mix*, Applied Biosystems, Carlsbad, California, USA). In a preliminary study (*22*), the reference gene was selected by geNorm software analysis (http://allserv.ugent.be/:jvdesomp/genorm/index.html) (*23*). Ribosomal protein S19 and glycerol-3-phosphate dehydrogenase (GPDH) genes exhibited the most stable constitutive expression patterns, followed by cyclophilin and actin (in crescent stability order). Efficiency of qPCR was analyzed using the software LinReg PCR Analysis of Real-Time PCR data, version 7.5 (*24*). Relative quantification (RQ) was determined by two distinct methodologies: the first one comparing the transcriptional expression between tagged genes and the constitutive reference gene S19 ($2^{-\Delta Ct}$) by a calculation formula derived from the $2^{-\Delta\Delta Ct}$ method ($\Delta = (Ct_{tag} - Ct_{ref})$, where Ct is the threshold cycle, tag = tag gene, and ref = reference gene) (*25*). The second one was used to evaluate the differential expression between two different conditions (genotype, location, and maturation stage) (*26*).

Primer design was based on the sequences obtained from the BCGP database using the software Primer Express (Applied Biosystems, Carlsbad, California, USA). Sequences of the tagged genes correspond to an orthologue of *MYO-INOSITOL(3)P1 SYNTHASE* from *A. thaliana*, two genes coding for PHOSPHATIDYL INOSITOL KINASE, and three *FERRITIN* genes, along with the reference gene ribosomal protein S19 (housekeeping) (**Table 1**).

Phytic Acid Determination. Phytic acid was determined in freeze-dried endosperms by reversed-phase high-performance liquid chromatography (*27*).

RESULTS

In Silico Analysis. *Phytate.* Structural metabolic pathways of phytate metabolism consist of two stages: the early pathways that comprise phytate synthesis and supply and the later inositol (Ins)-phosphate pathways that proceed either via soluble Ins-phosphates to Ins(3,4,6)P3 or via phosphatidyl-Ins phosphate intermediates to the production of Ins(1,4,5)P3 (*28*). The sole synthetic source of the Ins backbone is the activity of the enzyme *myo*-inositol(3)P1 synthase (MIPS) that converts glucose-6P to Ins(3)P1 (*29*). The pathway proceeds from the glucose derivative by phosphate addition via a family of protein kinases, namely, 3-kinase, 6-kinase, 4-kinase, 5-kinase, 1-kinase, and finally a 2-kinase.

Table 1. *Coffea arabica* (*Ca*) Primer Sequences for qPCR Analysis of the *Arabidopsis* and Maize Orthologue Genes for *MYO*-INOSITOL(3)P1 SYNTHASE (*CaMIPS1*), Two *PHOSPHATIDYL INOSITOL KINASE*-IP4 and IP5 2-Kinase (*CaIPK1*) and *INS* (*1, 3, 4*) *P3 5/6- KINASE* 1 (*CaIPK2*), Three Ferritin Genes (*CaFER1*, *CaFER2*, and *CaFER3*) and the Reference Gene Ribosomal Protein S19 (Housekeeping)

Name	Forward	Reverse
CaMIPS1	AAGCAATGTGGTGGATGACA	TGCTCCCCAGGTTCATAAAG
CaIPK1	TGGATTCTCCAAATGGTGCT	TGAGGTCAAAACTTTGGTTGG
CaIPK2	GGACCTCCGTTTGACACATT	CTGCAGGAGTTTGTGAACCA
CaFER1	CTGAAAAGCCGCACACTGC	TTGTTGGTATTTTCCTCATCTCAA
CaFER2	CATGCCCTGTTTGCCTATTT	GCAAGACCCTTGAGAGCAAC
CaFER3	TTACTTGAACGACATCAGATGAGG	TTTATTCCCTTGTTAATTTCCAATTC
S19	TGTCTTCAAATTCAGGACGCAC	CCAAACCCAGTTGACTTGCCT

In the *Coffea* transcriptome, 85 transcripts sharing sequence similarity to *A. thaliana* mRNAs involved in phytate metabolism were identified: 35 transcripts similar to those of the phytate-biosynthesis gene families (**Table 1**). As mentioned previously, MIPS catalyzes the first step of the *myo*-inositol pathway; therefore, a drastic reduction of phytate (94% and 68%) in soybean (*11*) and rice (*30*) seed contents was achieved by silencing *myo*-inositol-1-phosphate synthase genes (*GmMIPS1* and *RINO1*, respectively). In higher plants, the MIPS gene family consists of three to seven members, as seen in *Arabidopsis* (three) (*31*), soybean (four) (*32*), and maize (seven) (*33*). In *C. arabica*, one EST contig displaying sequence similarity to MIPS family genes was found; it shows 91% of amino acid identity to soybean *GmMIPS1* and consists of 42 reads mainly from the leaf (LV4, LV5, and LV8), floral bud (FB1 and FB2), and fruit libraries (FR1 and FR2). The full-length mRNA sequence was obtained, and the gene was labeled *CaMIPS1* (accession number GU108583). Furthermore, two singlet ESTs were identified; from a root (CA00-XX-RX1-086-A03-EB.F) and floral bud library (CA00-XX-FB1-105-D04-AC.F), displaying nucleotide identities of 69% and 82%, respectively, to *CaMIPS1*.

Stevenson-Paulik et al. (*34*) defined the molecular bases for the later steps of plant phytate biosynthesis studying *A. thaliana* transposon (T) mutants for *AtIPK1* (IP4 and IP5 2-Kinase) and *AtIPK2β* ([I(1,3,4,6)P4]5-kinase) genes. Seed extracts from *Arabidopsis* single *atipk1-1* and *atipk2β-1* mutants and the *atipk1-1 atipk2β-1* double mutant exhibited reduced seed phytate levels to 83%, 35%, and 95%, respectively. The *C. arabica* orthologue of *AtIPK1* was found as a unique contig, composed by two reads from floral buds (FB2) and embryogenic calli (EA1) libraries; the incomplete mRNA sequence was labeled as *CaIPK1* (accession number EZ421795). Protein identity and similarity between the coffee sequence and its *Arabidopsis* counterpart were of 50% and 69%, respectively. The partial protein sequence of *CaIPK1* displays the conserved boxes B, D, and E reported in plants and human IPK1 proteins (*35*).

Shi et al. (*36*) found out that the maize (*Zea mays*) low-phytic acid *lpa2* mutant is caused by a mutation in an inositol phosphate kinase gene *ZmIPK* (Ins (1,3,4)P3 5/6- kinase 1) and leads to a 30% reduction in phytic acid. The use of *ZmIPK* sequence to query *C. arabica* databases by BLAST searches provided the identification of two homologous contigs, C1 and C2 (accession numbers EZ421796 and EZ421797, respectively). The deduced amino acid sequence alignment analysis of *C. arabica* contigs and ZmIPK employing the ClustalW software showed 53% identity of the *C. arabica* contig C1 compared to that of ZmIPK, while the *C. arabica* contig C2 showed 39% and 38% of identity compared to those of ZmIPK and C1, respectively. The protein blast analysis between C1 and ZmIPK showed an e-value of 1e-52 and an identity of 45%. *Coffea* contig C1, assembled from two reads from floral buds (FB4) and fruit (FR2) libraries, has therefore been labeled as *CaIPK2*.

Six functional categories of phosphatidyl inositol kinases were also identified in coffee using gene ontology (molecular function): MORN domain (15%), 3−4 kinase (25%), 4−5 kinase (23%), Zn finger FYVE (18%), GroEL (15%), and triphosphate kinase (4%). Assignments are based on the data available at the TIGR *Arabidopsis thaliana* Gene Index version 13.0. Interestingly, the predominant forms of phosphatidyl inositol kinases corresponded to 3−4 and 4−5-dikinase. The functionally characterized MORN-domain family of phosphatidyl inositol kinase was abundant in *Coffea* libraries, mostly expressed in fruit-development and stress-response libraries (**Figure 1C**), which is consistent with its role in phytate accumulation and stress responses (*37*). The reverse pathway of phytate biosynthesis is carried out by a four-family group of protein phosphatases, synaptojanin (23%), monophosphatase (62%), polyphosphatase (7%), and endonuclease (8%). The most abundant group is the monophosphatase group that also presents higher levels of gene expression throughout the surveyed libraries and is highly expressed in floral buds and embryogenic calli cultures (**Figure 1A**). Thus, in silico evidence indicates that the *Coffea* protein phosphatase family plays nonredundant roles in phytate metabolism. Moreover, at this point, evidence suggest that the branching-point lies between stress induced and reserve phytate accumulation.

Ferritin. Four contigs exhibiting sequence similarity to *Arabidopsis* ferritin units were identified in *Coffea* databases: *CaFER1*, *CaFER2*, *CaFER3*, and *CaFER4* (accession number GQ913984, GU001880, EZ421798, and EZ421799, respectively). The sequences *CaFER1* and *CaFER2* correspond to complete mRNA sequences, while *CaFER3* and *CaFER4* represent incomplete transcript fragments, missing the 5′ ends. From the BCGP data, the main sources of ferritin-like transcripts correspond to suspension cells (RT8, CB1, and CS1), leaves (RM1), and stems (RX1) submitted to abiotic or biotic stress or hormone-treated floral buds (FB1 and FB4) and fruit libraries (FR1 and FR2) (**Figure 1C**). All transcripts (i.e., *CaFER1*, *CaFER2*, *CaFER3*, and *CaFER4*) were found in a library from flower buds at different developmental stages (FB4). The transcription levels of *CaFER1* and *CaFER2* were similar in the well-watered field plants (pool of tissues) (library SS1); however, *CaFER1* transcripts were highly abundant in suspension cells stressed with aluminum (library RT8) and in the stems infected with the *Xylella* libray (RX1) (**Figure 1B**). The ferritin gene family in *Coffea* shares restricted sequence similarity to its *Arabidopsis* counterparts. Phylogenetic analyses of the deduced amino acid sequences of complete *Coffea* sequences (CAFER1 and CAFER2) and other ferritin-like sequences from higher plants found in GenBank databases displayed tree major clusters: one formed by CAFER1 and ferritin sequences from *Solanum tuberosum* (gi|575804) and *Pyrus pyrifolia* (gi|89276797), a second one consisting of CAFER2 clustered with the vast majority of higher-plant sequences, including four *Arabidopsis* ferritin proteins, and the last one comprising ferritin sequences from the leguminous family, soybean (*G. max*) and *Medicago sativa* (**Figure 2**).

Phytate Contents. Phytic-acid seed contents for *Coffea* cherries of cultivars Catuai Amarelo and Obatã at G1, CR1, and CR2

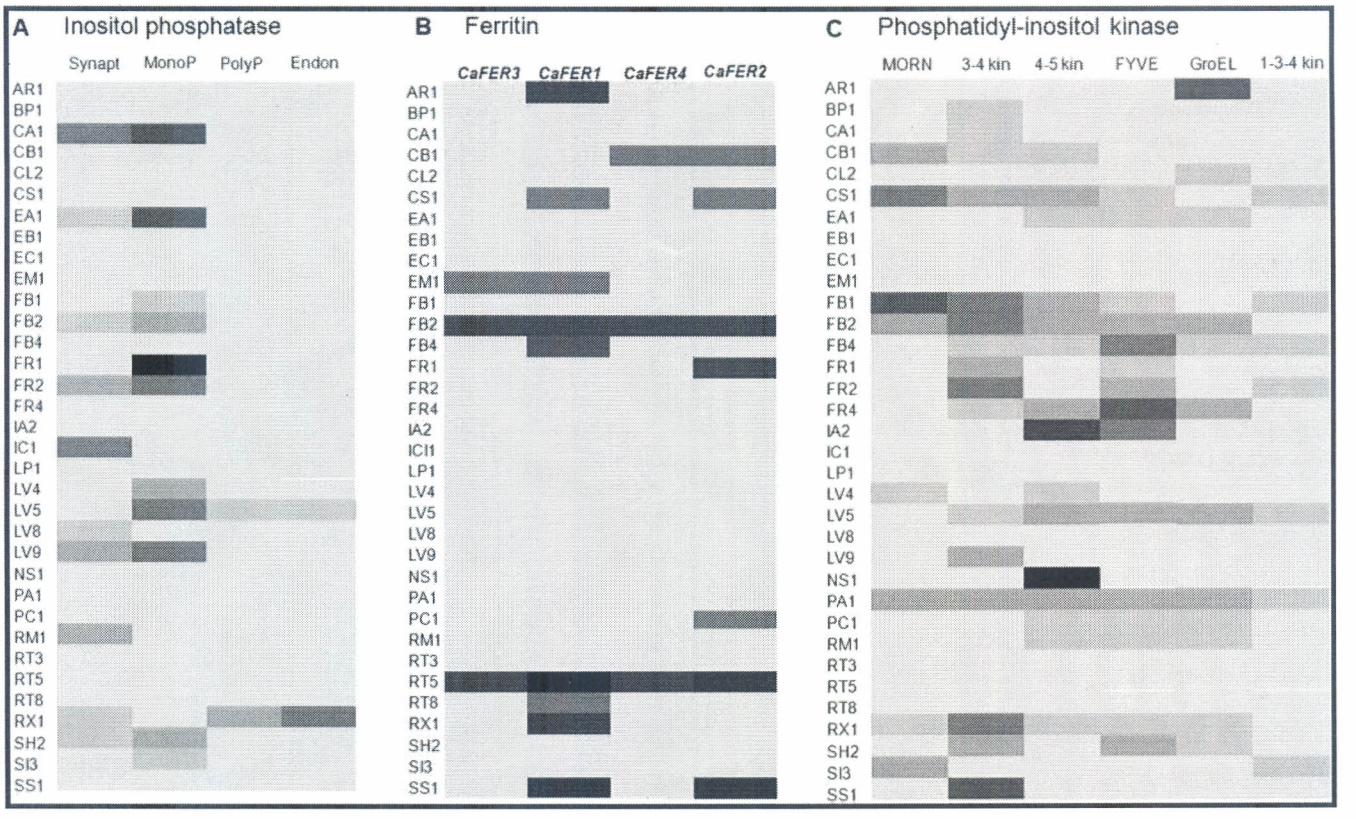

Figure 1. In silico expression profile (**A**): inositol phosphatase; (**B**) *CaFER1*, *CaFER2*, and *CaFER3*; (**C**) phosphatidyl-inositol kinase family. Synap, synaptojanin; MonoP, monophosphatase; PolyP, polyphosphatase; Endon, endonuclease. The normalized number of reads for the transcripts in each library is represented as a grayscale. *Coffea* libraries are represented as lines, and the gene names are shown as columns. Library abbreviations correspond to the following descriptions: AR1, leaves treated with arachidonic acid; BP1, suspension cells treated with acibenzolar-*S*-methyl; CA1, nonembryogenic calli; CB1, suspension cells treated with acibenzolar-*S*-methyl and brassinosteroids; CL2, hypocotyls treated with acibenzolar-*S*-methyl; CS1, suspension cells under osmotic stress; EA1, *Coffea arabica* embryogenic calli; EB1, zygotic embryos from immature fruits; EC1, *Coffea canephora* embryogenic calli; EM, zygotic embryo from mature germinating seeds; FB1, floral buds at developmental stages 1 and 2; FB2, floral buds at developmental stages 1 and 2; FB4, floral buds at developmental stages 3 and 4; FR1, floral buds, pinhead fruits, fruit developmental stages 1 and 2; FR2, floral buds, pinhead fruits, fruit developmental stages 1 and 2; FR4, *C. racemosa* fruits; FV2, *C. racemosa* fruits at developmental stages 1, 2, and 3; IA2, *C. arabica* embryogenic cell line induced with 2,4-dichlorophenoxyacetic acid; IC1, *C. arabica* nonembryogenic cell line without 2,4-dichlorophenoxyacetic acid; LP1, plantlets treated with arachidonic acid; LV4, young leaves from orthotropic branches; LV5, young leaves from orthotropic branches; LV8, mature leaves from plagiotropic branches; LV9, mature leaves from plagiotropic branches; NS1, nematode-infected roots; PA1, primary embryogenic *C. arabica* calli; PC1, *C. arabica* nonembryogenic cell line induced with 2,4-dichlorophenoxyacetic acid; RM1, leaves infected with leaf miner and coffee leaf rust; RT3, roots; RT5, roots with with acibenzolar-*S*-methyl; RT8, root suspension cells under aluminum stress; RX1, *Xyllela* spp.-infected stems; SH2, water-stressed plant tissues; SH3, water-stressed *C. canephora* leaf tissue; SI3, germinating whole seeds; SS1, well-watered field plant tissues.

developmental stages grown in Ouro Fino and the CR1 stage from Adamantina, analyzed by HPLC, are shown in **Figure 3**. The seeds exhibit no significant differences in phytic acid contents between cultivars and locations; in mature seeds, the contents ranged from 2.1 mg to 2.4 mg of phytic acid per gram of seed (mg/g) (**Figure 3**). Seeds at developmental stage G1 displayed an approximate 3-fold reduction in phytic acid in comparison to that of CR2 cherry seeds for both cultivars. The berries at the later maturation stage (CR2) showed a 10% increase in phytic acid contents in comparison to that of the previous maturation stage investigated, CR1.

Relative Quantification (RQ) by qPCR. *Phytate Biosynthesis.* On the basis of in silico results, *Coffea* orthologues of well-characterized *Arabidopsis*, soybean, and maize genes involved in phytate biosynthesis were selected for qPCR analysis; *CaMIPS1*, *CaIPK1*, and *CaIPK2*. We have investigated the transcriptional regulation of these genes in seeds at four maturation stages (i.e., G1, G2, CR1, and CR2) from plants grown in Ouro Fino and at two stages (G1 and CR2) from plants grown in Adamantina, using two commercial cultivars of *C. arabica*: Catuai Amarelo and Obatã.

The transcripts corresponding to *CaMIPS1*, *CaIPK1*, and *CaIPK2* exhibited a similar expression pattern for the fruit developmental stages, cultivars, and locations tested. However, RQ analyses of the *CaMIPS1* transcriptional profile demonstrated the presence of developmental regulation: in Obatã, *CaMIPS1* was up-regulated at the G1 maturation stage, with approximately 6-fold, 3-fold, and 5.7-fold overexpression related to G2, CR1, and CR2, respectively (Table **3**). Catuai seeds exhibited an approximately 3-fold increase in *CaMIPS1* transcripts at G1 stage in comparison to G2 (**Table 3**). No significant difference was found for *CaMIPS1* expression between cultivars (i.e., Obatã and Catuaí Amarelo) and locations (i.e., Adamantina and Ouro Fino) (**Table 3**). Similarly, no significant difference in *CaIPK1* expression was observed for the investigated maturation stages (i.e., G1, G2, CR1, and CR2) and between cultivars (i.e., Obatã and Catuaí Amarelo) and locations (i.e., Adamantina and Ouro Fino) (**Table 3**). A slight RQ difference was identified in *CaIPK2* expression levels for the tested locations, cultivars, and developmental stages (**Table 3**). The most significant difference was found in comparing fruit maturation: Obatã fruits at the G1 stage showed approximately 2.5-fold up-regulation in *CaIPK2* transcripts in comparison to that of G2.

Ferritin. From the four contig homologues to ferritin genes found in the *Coffea* transcriptome database, it was possible to design specific primers for qPCR analyses for two contigs, *CaFER2* and *CaFER3*. The high nucleotide identity between *CaFER1* and *CaFER4* (98%) prompted us to design effective primers based on the *CaFER1* sequence, which consisted of a more representative number of reads.

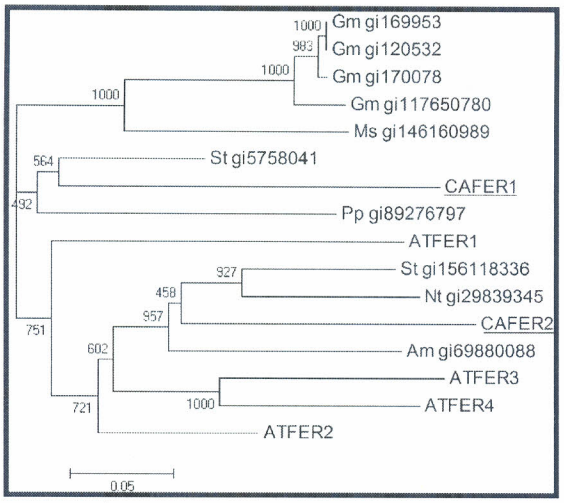

Figure 2. NJ tree generated by ClustalX alignment of coffee (CAFER1, CAFER2, and CAFER3) deduced amino-acid sequences homologues to the *Arabidopsis* ferritins and full-length sequences from the protein database at NCBI. Genbank accession number is shown next to the species abbreviation. Ca, *Coffea arabica*; At, *Arabidopsis thaliana*; Gm, *Glycine max*; Ms, *Medicago sativa*; St, *Solanum tuberosum*; Av, *Avicennia marina*; Nt, *Nicotiana tabacum*; Pp, *Pyrus pyrifolia*. Ferritins *Arabidopsis* accession number: ATFER1 (AF229850) ATFER2 (AC009991) ATFER3 (AL163763) ATFER4 (AF085279).

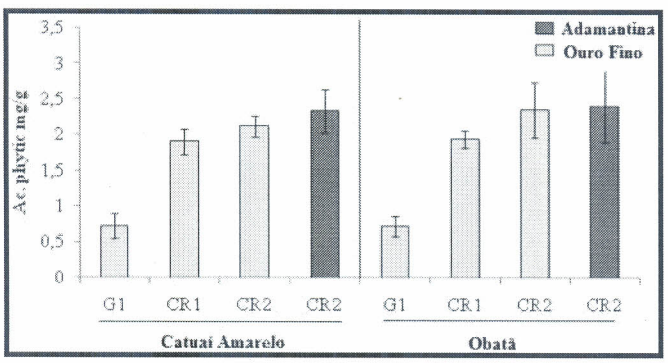

Figure 3. Analysis of coffee seed contents of phytic acid (in mg per gram of seed, mg/g) by HPLC. The cultivars Catuaí Amarelo, and Obatã were analyzed at the development stages: green stage enclosing completely milky endosperm (G1), cell elongation of endosperm (CR1), and second cherry stage with remaining outer layer perisperm silver or silver skin pericarp maturation (CR2). Coffee plants growing in Ouro Fino and Adamantina. The error bars represent the standard deviation calculated for three samples (same genotype, trees growing at the same plot).

The expression profile of three ferritin units (*CaFER*1, *CaFER*2, and *CaFER*3) was distinct (**Figure 4**). In both investigated cultivars, the expression of *CaFER*3 was low during fruit maturity stages, in comparison to those of *CaFER*1 and *CaFER*2 (**Figure 4**). This result is in agreement with the observations from in silico analyses (**Figure 1B**). The differences in the transcriptional profile of *CaFER1* and *CaFER2* are evident at the CR2 and G2 stages; in the first stage, the expression levels of *CaFER2* was higher than that of *CaFER1* (Catuaí Amarelo, Ouro Fino; Catuaí Amarelo, Adamantina; Obatã, Adamantina), except for Obatã fruits from Ouro Fino. In contrast, at the following stage (G2), *CaFER1* expression levels were higher than that of *CaFER2* (Catuaí Amarelo and Obatã, Ouro Fino).

Analyses of RQ at the maturation stage (**Table 3**) demonstrated that *CaFER1* expression was higher in G1 than in CR1 and CR2. The transcripts corresponding to *CaFER1* exhibited an approximately 7-fold and 4-fold increase at G1 in comparison to that at CR1 in Catuaí Amarelo and Obatã, respectively, in Ouro Fino (**Table 3**). At the CR2 developmental stage in Obatã plants, *CaFER1* expression exhibited an approximately 19-fold increase in Ouro Fino, in comparison to the levels observed in Adamantina. Interestingly, no significant differences were found for Catuaí Amarelo seeds in both locations. Comparing both cultivars at the same location, *CaFER*1 exhibited an approximately 5-fold induction in Obatã plants at the CR2 stage in comparison to that of Catuaí Amarelo in Ouro Fino. In contrast, for plants grown in Adamantina, *CaFER*1 displayed a 5-fold increase in expression at the CR2 stage for Obatã relative to that of Catuaí Amarelo.

Results from RQ analyses of *CaFER*2 expression for Catuaí Amarelo and Obatã seeds from plants grown in Ouro Fino are shown in **Table 3**. The expression of *CaFER*2 was induced at G1 in comparison to G2, CR1, and CR2 for both cultivars (i.e., Catuai Amarelo and Obatã). The RQ difference for *CaFER2* expression from plants grown in Adamantina and Ouro Fino was significant for Catuaí Amarelo seeds at the G1 stage; it displayed approximately 3-fold induction in Ouro Fino in comparison to that in Adamantina, whereas Obatã seeds at the CR2 stage exhibited a 5-fold induction of *CaFER2* expression in Adamantina in comparison to that in Ouro Fino (**Table 3**). *CaFER2* expression was 5-fold induced in Catuaí Amarelo in comparison to that in Obatã for fruits at CR2 in Ouro Fino (**Table 3**).

DISCUSSION

Phytate. The phytic acid contents in coffee seeds harvested from the two locations (i.e., Ouro Fino and Adamanina) and two cultivars (i.e., Obatã and Catuai Amarelo) displayed no

Table 2. Phytate and Ferritin ESTs from the *Coffea* Database (BPGC) Search Using the *Arabidopsis* (*At*) Homologues as Reference

gene name	*At* protein code	% similarity (similarity, coverage)	functional domain and biological process	ref
FIERY1	AT4G21670	11 contigs and singlets (99 to 12.7%, FL to 27.0%)	bisphosphate nucleotidase, double stranded RNA binding, phytate synthesis	*37*
ferritin family	AT2G40300	4 contigs	Ferritin, iron ion homeostasis, iron ion transport, response to iron ion	*10*
IK family	AT3G09920	25 contigs and singlets (100 to 24.5%,/FL to 33.0%)	inositol kinase	*52*
IP X PI family	AT1G34120	10 contigs and singlets (100 to 19.0%, 85 to 21.0%)	inositol monophosphatase	*53*
MIOX family	AT1G14520	2 contigs (60.2 to 17.7%,/87 to 23%)	*myo*-inositol oxidase	*54*
*At*PIS1/PGP family	AT1G68000	35 contigs and singlets (100% to 24.5,/FL to 33%)	phosphatidyl glycerol phosphate synthase	*55*
PCP family	AT1G07230	2 contigs (95 to 24.4%/FL to 25%)	phospholipase C	na[a]

[a] na: not available.

Table 3. Relative Quantification (RQ) of mRNA Corresponding to Phytate Biosynthesis Genes *CaMIPS1*, *CaIPK1*, and *CaIPK2* and Two Ferritin Genes, *CaFER1* and *CaFER2*, in Different Stages of Developing Seeds Using the Method Devised by Pfaffl et al. (*24*)[a]

	CaMIPS1	*CaIPK1*	*CaIPK2*	*CaFER1*	*CaFER2*
			G1 in comparison to fruit maturation		
Catuaí Amarelo: Ouro Fino					
G1	1.00	1	1.00	1.00	1.00
G2	−3.08 ± 1.19	1.10 ± 0.20	−1.85 ± 0.65	−1.16 ± 0.45	−11.1 ± 2.7
CR1	−1.61 ± 0.12	−1.07 ± 0.14	2.02 ± 0.22	−6.75 ± 2.73	−3.11 ± 0.48
CR2	1.17 ± 0.24	−1.27 ± 0.57	−2.02 ± 1.69	−8.08 ± 5.06	−3.69 ± 1.97
Obatã: Ouro Fino					
G1	1.00	1.00	1.00	1.00	1.00
G2	−6.17 ± 3.96	−1.97 ± 1.22	−2.49 ± 1.46	−1.36 ± 0.31	−8.74 ± 4.91
CR1	−2.96 ± 0.82	−1.25 ± 0.15	−1.48 ± 0.92	−4.23 ± 2.21	−2.23 ± 0.26
CR2	−5.68 ± 3.63	−1.72 ± 1.01	−1.16 ± 0.81	−1.83 ± 0.54	−8.03 ± 3.32
			Adamantina in comparison to Ouro Fino		
Catuaí Amarelo					
G1	−1.39 ± 0.42	−1.21 ± 0.34	−1.99 ± 1.19	1.21 ± 0.21	−2.90 ± 1.26
CR2	−1.08 ± 0.19	−1.21 ± 0.09	1.08 ± 0.20	1.18 ± 0.79	1.48 ± 0.27
Obatã					
CR2	1.84 ± 0.23	−1.08 ± 0.09	−2.11 ± 0.50	−19.0 ± 18.2	5.18 ± 1.17
			Catuaí Amarelo in comparison to Obatã		
Ouro Fino					
G1	−1.21 ± 0.12	1.56 ± 0.04	1.15 ± 0.10	−2.42 ± 1.23	−1.94 ± 0.59
G2	1.47 ± 0.57	2.13 ± 0.39	1.48 ± 0.52	−1.77 ± 0.97	1.21 ± 0.06
CR1	1.50 ± 0.11	1.75 ± 0.20	2.28 ± 0.25	−1.97 ± 0.80	1.51 ± 0.25
CR2	1.53 ± 1.13	1.87 ± 0.74	−2.02 ± 1.70	−4.97 ± 3.11	5.01 ± 2.30
Adamantina					
CR2	1.29 ± 0.15	1.51 ± 0.11	1.09 ± 0.20	5.51 ± 3.70	1.78 ± 0.32

[a] The ribosomal protein S19 constitutive gene was used as reference. Development green stage with liquid endosperm tissue (G1); green stage enclosing the completely milky endosperm, endosperm cell elongation (G2); first cherry stage with remaining outer layer perisperm still green (CR1); second cherry stage with remaining outer layer perisperm silver or silver skin, pericarp maturation (CR2). Means of three biological replicates ± standard deviation.

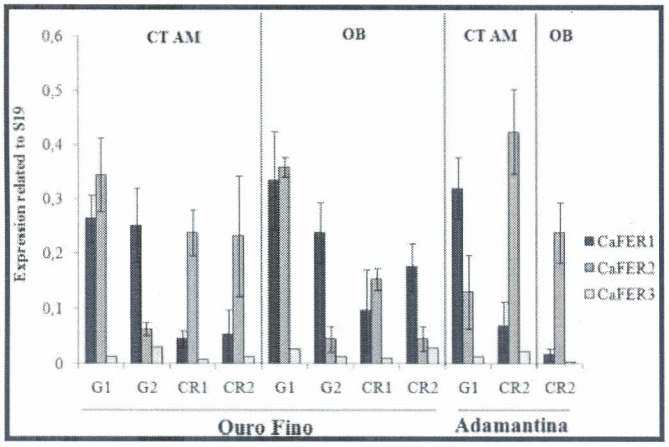

Figure 4. Quantitative PCR analysis of the mRNA from the ferritins coffee genes *CaFER1*, *CaFER2*, and Ca*FER3* in comparison to the constitutive *S19* gene, using the calculation $2^{-\Delta Ct}$, a derivate from the $2^{-\Delta\Delta Ct}$ method (*23*). Green stage with a liquid endosperm tissue (G1); green stage with completely milky endosperm, endosperm cell elongation (G2); first cherry stage with the remaining outer layer perisperm green (CR1); second cherry stage with the remaining outer layer perisperm silver or silver skin, pericarp maturation (CR2). The error bars represent the standard deviation calculated for three samples (same genotype trees, growing at the same plot). CT AM, Catuaí Amarelo; OB, Obatã.

significant differences. Phytic acid contents increased during fruit maturation in cultivars Obatã and Catuaí Amarelo until the fruits reached the cherry stage. A similar profile of phytic acid accumulation throughout seed development was observed for soybean, where a steady increase in the compound levels was observed until late seed maturation (*38*). In important grain crops, such as soybean, maize, and rice, the average levels of phytic acid in seeds are approximately 3.0 mg/g (*28*). In *Arabidopsis thaliana*, Bentsink et al. (*39*) analyzed the natural variation of phytic acid contents and Pi accumulation in seeds and leaves for a large number of accessions of *Arabidopsis*, and their work revealed a wide range of variation in phytate contents, from 7.0 mg to 23.1 mg/g of seeds. Phytic acid contents in coffee beans has been established in a range of 1.2 to 5.4 mg/g of seeds (*40*), occurring in mature fruits with 3−6 mg/g dry weight (*41*). In the present work, phytic acid levels for the cultivars Obatã and Catuaí Amarelo ranged from 2.1 to 2.4 mg/g. The quality of the beverage produced by beans from the two localities where the fruits were collected has been consistently characterized as different. Adamantina produces lower quality beans (*42*) probably due to a delay of up to three months in fruit ripening in comparison to that in Ouro Fino. The delay in fruit ripening and the coffee beverage quality have been associated with the biochemical composition under distinct environmental conditions, e.g., the exposure to light (*43*, *44*), warmer temperatures, (*42*) and altitude (*45*). The mechanisms underlying temperature effects on coffee quality remain elusive, but coffee farmers empirically speculate that lower temperatures allow the maturation to occur at a slower pace permitting quality to develop, possibly due to the kinetics of the biochemical reactions in the beans. Vaast et al. (*43*) suggested that higher sucrose, chlorogenic acid, and trigonelline contents in sun-grown beans are related to incomplete bean maturation, thus being responsible for the higher bitterness and astringency of the coffee beverage. During the investigated period, the difference between the highest and lowest mean annual temperatures in Adamantina and Ouro Fino were 4.7 and 4.3 °C, respectively.

Concerning the altitude difference, Adamantina is 749 m lower than Ouro Fino in relation to the average sea level. Despite the differences regarding the environmental conditions, plants grown in Adamantina and Ouro Fino did not exhibit significantly distinct phytate contents. In accordance to the data on phytic acid contents, the transcriptional level of *CaMIPS1*, *CaIPK1*, and *CaIPK2* in two coffee cultivars grown in two environmentally distinct locations showed no significant differences. Contents of *myo*-inositol in coffee grains were analyzed during grain development in three cultivars of *C. arabica* and two of *C. canephora*, showing considerable amounts of *myo*-inositol (30−40 mg/g dry weight) in young grains, whereas only the phosphorylated form of phytic acid occurred in mature grains at 3−6 mg/g dry weight (*41*). Recently an elegant work investigating the metabolic pathway in coffee grains showed that *myo*-inositol contents and *CaMIPS1* transcripts exhibit high levels at the initial phase of grain development, also displaying substantial amounts in the transient perisperm (*46*). *myo*-inositol contents and *CaMIPS1* transcripts exhibited an abrupt drop coinciding with the beginning of endosperm development, and the decrease was stabilized when the endosperm reached the perisperm dry mass similar to that found in the grain (*46*). The contents of *myo*-inositol and *CaMIPS1* transcript levels decline at a slight ratio when the endosperm developed rapidly and replaced the perisperm (*46*). These observations are compatible with the transcriptional profile of *CaMIPS1* observed in our study since a slight up-regulation of *CaMIPS1* expression was observed at the green stage with the endosperm at the milky stage (G1), which corresponds to rapidly developed endosperm replacing perisperm. Raboy and Dickinson (*38*) studied phytic acid accumulation in developing soybean seeds and showed that levels increased steadily until later stages of seed maturation, throughout two phases of phosphate (P) metabolism. The period of cell division and growth is devoted to the synthesis of P compounds required for processes, such as nucleic acid metabolism, membrane phospholipid biosynthesis, etc., so that at these stages only traces of phytic acid are present. Subsequentially, phytic acid accumulation is initiated, and the accumulation rate of other P compounds declines to negligible amounts during a brief transition period. Thereafter, a steady rate of phytic acid accumulation is maintained until very late in seed maturation (*38*). The progressive accumulation of phytate is supposed to be mediated by a transporter. Shi et al. (*12*) identified an ATP-binding cassette (ABC) transporter as a key contributor to phytic acid accumulation in maize and soybean seeds. In contrast to the *CaMIPS1* profile, mRNA levels of *CaIPK1* and *CaIPK2* exhibited a tendency to steady state. The profile of *CaIPK1* and *CaIPK2* gene expression during the development of coffee seeds indicates that phytate biosynthesis is regulated independently of phytate accumulation, in agreement with evidence of the involvement of transporter activity to increase the compound levels (*12*).

myo-inositol has ambiguous uses in several functions at the cellular level since *myo*-inositol phosphates are precursors of several metabolic pathways (*29*). The ubiquitous biological role of the molecule could account for the induced expression of *CaMIPS1* during the early phases of coffee fruit development where the contents of phytic acid are still low.

Currently, it is not clear as to how many and which members of the *MIPS* gene family contribute to phytate synthesis due to the high levels of sequence similarity. In rice, silencing the gene *MIPS* (*RINO1*) at later stages of seed maturation resulted in a 68% reduction in phytate content (*30*). The accumulation of *RINO1* transcripts in seeds coincided with the presence of phytin-containing particles in the embryos and aleurone layers (*47*). More recently, Suzuki et al. (*48*) confirmed the specific expression pattern of *RINO1* by qPCR, in contrast to its homologous gene *RINO2* that was mainly expressed in anther tissues. In soybean, data from Hegeman et al. (*32*) indicate that a single gene, *GmMIPS1*, is expressed in immature cotyledons. Similarly, Nunes et al. (*11*) have demonstrated that the knockout of *GmMIPS1* caused a decrease of 93% of the phytate contents in soybean grains. Although interesting, the study did not conclusively show that the whole *GmMIPS* family was silenced since at least four *MIPS*-encoding sequences, displaying a high degree of sequence similarity, are present in the soybean genome. In contrast, three *MIPS* genes have been identified in *Arabidopsis* (*AtMIPS1*, *AtMIPS2*, and *AtMIPS3*), and all of them were expressed not only in siliques but also in leaves and roots (*31*). Moreover, qPCR analyses revealed that the relative expression of *AtMIPS1* and *AtMIPS2* in developing siliques was higher than that of *AtMIPS3* (*31*). Transcripts corresponding to MIPS in *Arabidopsis* were found in the endosperm and at phytin-containing particles in the embryos (*31*). In silico analyses of the *MIPS* gene family in *Coffea* allowed us to identify one *MIPS* family gene (*CaMIPS1*), which was highly expressed in several coffee tissues. Similarly, two contigs representing rare transcripts were found in root and floral bud libraries. Currently, it remains inconclusive whether other *MIPS* family genes participate, along with the characterized *CaMIPS1*, in phytate synthesis in coffee seeds.

Reduced contents of phytic acid usually have negative effects on seed and plant performance (*6*). Recently, two successful approaches in maize, soybean (*12*), and rice (*30*) reduced the compound levels by introducing gene-silencing constructs driven by embryo-specific promoters from either oleosin or globulin-1 genes, thus avoiding the negative effects of reduced phytic acid contents in other plant parts. While in the first study a locus encoding an ABC transporter, probably responsible for the transport and compartmentalization of phytate in the vacuoles, was silenced (*12*), in the later study, the silenced gene corresponded to the enzyme RINO1 (*30*).

Ferritin. Our data have demonstrated the presence of two important ferritin units expressed during the development of coffee seeds, *CaFER1* and *CaFER2*. In *Arabidopsis*, *AtFER2* has been demonstrated to have a maturity seed-specific expression profile (*6*, *43*). In contrast, in *Coffea*, no maturity-specific ferritin unit was identified. In silico analyses revealed a ubiquitous expression pattern of *CaFER1* and *CaFER2*. In general, *CaFER2* exhibited higher expression levels in mature seeds (CR2) than *CaFER1*, with the exception of seeds from the cultivar Obatã in Ouro Fino. Interestingly, transcriptional analyses by Petit et al. (*7*) found that *AtFER2* is exclusively expressed in *Arabidopsis* seeds, whereas similar results were obtained by proteomic assays carried out by Ravet et al. (*49*). In coffee, our transcription data indicate that at least two members of the family, *CaFER1* and *CaFER2*, are expressed in mature seeds. However, at this point, the presence of further ferritin unit genes in *Coffea* cannot be ruled out. Moreover, the existence of alternate mechanisms controlling ferritin accumulation remains to be further investigated in *Coffea*, as shown for *Arabidopsis* by the presence of post-translational regulation of ferritin accumulation and the effect of the balance of iron allocation between vacuoles and plastids in ferritin stability (*49*).

Although *CaFER1* and *CaFER2* exhibited low levels of nucleotide and protein sequence similarity, 44.9% and 50.7%, respectively (data not show), both were phylogenetically clustered with plant ferritin units associated with oxidative stress. CAFER1 is phylogenetically related to *Solanum tuberosum* ferritin (*StF1*), whose transcripts accumulate in response to pathogen attack in leaves and to elicitor (eicosapentaenoic acid) treatment in

tubers (*50*). The authors suggested that H_2O_2 release, triggered by the pathogen and elicitor, was responsible for increasing ferritin expression (*50*). In contrast, CAFER2 is phylogenetically close to *Avicennia marina Fer1*, which plays a role in oxidative stress responses, by environmental conditions and genetic background induced by iron, light stress, and directly by H_2O_2 treatment, as confirmed by transcriptional analyses (*51*). In *Coffea*, the distinct expression pattern observed for the ferritin genes (*CaFER1* and *CaFER2*) in both investigated locations (i.e., Adamantina and Ouro Fino) and cultivars (Obatã and Catuai) is likely to reflect distinct environmental stress conditions to which the plants are submitted.

Concluding Remarks. Conventional coffee breeding programs often associate the beverage quality with locally existing environmental conditions. Associating metabolic and transcriptional data, we found that the investigated environmental conditions did not cause significant alterations in phytate biosynthesis, whereas the expression profile of ferritin unit genes is affected by environmental conditions and genetic background.

The current work represents the application of genomic and metabolomic tools to help understand complex traits sought after by coffee breeding programs, such as beverage quality, specifically concerning iron biofortification and low phytic acid contents. In silico analyses and transcriptional profiling have revealed *CaMIPS1*, *CaIPK1*, and *CaIPK2* as candidate genes involved in phytate biosynthesis in coffee seeds. The biological functions of these candidates remain to be further investigated so that approaches to obtain low phytic acid content in coffee grains using molecular breeding can be devised, such as spatially and temporally regulated gene silencing using promoter-specific gene constructs. Similarly, interesting candidates for the role of key regulators in ferritin metabolism have been identified for coffee seeds, although the post-translational mechanisms and other regulatory feedback loops involved in ferritin accumulation remain to be further investigated.

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