Comparative proteomic analysis between early developmental stages of the *Coffea arabica* fruits

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ABSTRACT. Coffee is one of the most valuable agricultural commodities. There is much agronomic research on coffee, but molecular knowledge of its fruit development and ripening is limited. This study reports a comparative proteomic investigation of immature coffee fruits in two early developmental stages: stage 1, cell division and elongation of the perisperm; and stage 2, early growth of the endosperm progressively replacing the perisperm. Proteins were extracted using a modified SDS-phenol method and two-dimensional electrophoresis gels stained with

Coomassie blue revealed about 300 well-resolved polypeptide spots in the pH range of 3 to 10. The differentially expressed polypeptides spots were excised, trypsin-digested, and analyzed by MALDI-TOF mass spectrometry. Peptide MS data were searched against the coffee EST database. Most of the identified protein spots are involved in the glycolytic pathway and energy reserve, and are more highly expressed at stage 2.

Key words: Coffee; Growth; Immature fruit; Proteome

INTRODUCTION

Coffee is an important agricultural commodity produced in more than 60 countries worldwide. Brazil alone accounts for more than one third of the global coffee production and exportation (Vieira et al., 2006). The two main species cultivated in the world are *Coffea arabica*, which represents approximately 70% of world production, and *Coffea canephora*, which represents the remaining 30% (Geromel et al., 2006). Because of the economic importance of coffee, a great amount of agronomic work has been done (Rogers et al., 1999a) such as on flowering, yield, bean size, cup quality, caffeine content, disease, and drought resistance (Vieira et al., 2006). Most of these studies focused on ripe fruit components such as caffeine, chlorogenic acids, trigonelline, diterpene, sucrose, and phenolic compounds (Mazzafera and Robinson, 2000; De Castro and Marraccini, 2006; Geromel et al., 2006). Furthermore, studies about changes in concentration of mono- and oligosaccharides, sugar alcohols, myo-inositol, carboxylic acids, inorganic anions (Rogers et al., 1999b), and ethylene in coffee during grain development were also reported (Pereira et al., 2005). The protein composition of the fruit was also the object of some coffee studies, because they are precursors of nitrogen compounds involved in the fruit flavor (Rogers et al., 1999a; Estanislau, 2002). Despite the wide spectrum of agronomic studies, the molecular knowledge of fruit development and ripening is still limited. A study of the coffee seed development using proteomics and two-dimensional (2-D) gels was reported recently. It focused on zygotic embryos at two later developmental stages, 210 and 255 days after anthesis (DAA), since coffee seed development is accompanied by severe modifications in water-soluble proteins and several of these are associated with a specific developmental stage (Franco et al., 2009).

Fruit development and ripening studies are important, because the pathways involved in these processes are unique to plants and vary between species. Although different species share common pathways and developmental programs, the physiological, anatomical, and biochemical compositions and structural differences must contribute to the operation of unique pathways, genes, and proteins (Katz et al., 2007). The elucidation of the molecular basis of such early and common events represents an active frontier in fruit ripening research (Giovannoni, 2004). Lemaire-Chamley et al. (2005) analyzed genes expressed from the early developing fruit of a tomato, using microarrays, and found a higher proportion of genes with unknown functions, suggesting that future studies on early tomato fruit development should preferentially focus on this stage, which is concomitant with the transition from cell division to cell expansion. Through the scale of the coffee fruit phenological stages, proposed by Pezzopane et al. (2003), cell division and cell expansion stages can be visually observed. The cell division and elongation of the perisperm stage, at 0 to 60 DAA, is characterized by intense cell division but with no change in fruit size (Pezzopane et al., 2003; De Castro and Marraccini, 2006). This absence of growth is associated with high levels of abscisic acid and low levels of gibberellic

acid (Thomaziello et al., 2000). The cell expansion stage, at 60 to 90 DAA, is characterized by a rapid increase in fruit diameter, with gradual disappearing of the transient perisperm and the early endosperm expanding (Cutter, 1987; De Castro and Marraccini, 2006; Joët et al., 2009).

In this study, the proteomic profiles of two developmental stages of immature coffee (55 and 90 DAA) were compared to identify differentially expressed proteins and relate them to the metabolic pathways involved. The results may contribute to future studies about fruit development and growth using molecular strategies.

MATERIAL AND METHODS

Plant materials

C. arabica fruits of the IAPAR 59 cultivar were collected at two early developmental stages: stage 1 at 55 DAA, and stage 2 at 90 DAA. Stage 1 encompasses 50 to 60 DAA, which corresponds to the cell division and elongation of the perisperm, and stage 2 encompasses 85 to 95 DAA, which corresponds to the early growth of the endosperm progressively replacing the perisperm. Immediately after collection, the fruits were frozen in liquid nitrogen and stored at -80°C until analysis.

Preparation of total protein extracts

Total proteins were extracted from the fruits according to the modified sodium SDS-phenol method described by Wang et al. (2003). Grains were finely ground in a liquid nitrogen pre-cooled mortar by using a pestle. Approximately 2 g of the resulting tissue powder (20 mL) was placed into a centrifuge tube and suspended in 20 mL extraction buffer I [containing 1% polyvinyl-polypyrrolidone, 2% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) in acetone]. The mixture was vortexed thoroughly for 30 s, kept on ice, and sonicated for 20 s at 30% output of the sonicator (Fisher Scientific Sonic Dismembrator model 500). Samples were centrifuged (15 min, 5525 g, 4°C) and the supernatant was discarded. The resulting pellet was washed twice with 15 mL ice-cold acetone, and each washing step was centrifuged at 5525 g for 5 min at 4°C. The pellet was resuspended three times in 15 mL of cold 10% trichloroacetic acid (TCA) in acetone. After that, it was washed twice with cold aqueous 10% TCA and twice with cold 80% acetone. A final wash was performed with cold 80% ethanol. Each time, the pellet was resuspended completely by vortexing and was then centrifuged as described above. The final pellet was dried at room temperature for about 16 h.

The pellet obtained was resuspended in 10 mL extraction buffer II (containing 30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8, 2% 2-mercaptoethanol, and 1 mM PMSF) and immediately vortexed thoroughly for 30 s. It was then kept at room temperature for 20 min. Then, 12 mL Tris-HCl saturated with phenol, pH 8, was added, and the mixture was vortexed thoroughly three times for 40 s each. The sample was then incubated on ice for 5 min. The phenol phase was separated by centrifugation at 5525 g for 5 min at 4°C. The upper phenol phase was collected in centrifuge tubes (approximately 10 mL). Proteins in the phenol phase were precipitated in 3 volumes of cold 0.1 M ammonium acetate in methanol at 20°C for 40 min. Precipitated proteins were recovered through centrifugation at 5525 g for 10 min at 4°C, washed twice with 15 mL cold 0.1 M ammonium acetate, and then centrifuged again at 2000 g for 5 min at 4°C. The resulting pellet was washed twice with 15 mL cold 0.1 M ammonium acetate, three times

with cold 80% acetone, and once with cold 80% ethanol. After each washing, the pellet was resuspended completely by vortexing and then centrifuged at 5525 *g* for 5 min at 4°C.

The resulting pellet was dried at room temperature for about 24 h, and then was dissolved in 400 μL buffer [7 M urea, 2 M thiourea, 4% CHAPS, and 40 mM dithiothreitol (DTT)] and stored at -20°C. The total protein content was estimated according to Bradford (1976).

Two-dimensional electrophoresis

Isoelectric focusing and molecular mass separation were conducted using an IPGphor II and an SE 600 Ruby according to the manufacturer protocol (GE Healthcare). Approximately 600 μg protein was applied on 11-cm strips with a linear pH range from 3 to 10 (GE Healthcare). Isoelectric focusing was performed at 20°C, with cur¬rent limited to 50 μA/strip and applying 500 (4 x), 800, 7000, and 3700 Vh. The strips were equilibrated in buffer (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, and 0.002% bromophenol) containing 1% DTT (w/v) for 15 min, followed by a 15 min incubation in the same buffer without DTT and containing 2.5% iodoacetamide (w/v) according to GE Healthcare. The second dimension was carried out on an 18 x 18 cm 11.7% gel via SDS-PAGE. The gels were stained with Coomassie blue R250.

Gel image analysis

Images were captured by scanning the gels with an Image Scanner (GE Healthcare) and LabScan software (GE Healthcare) and were analyzed by the ImageMaster2D Platinum 6.0 software (GE Healthcare). Three gels were analyzed per sample. The gels that presented more spots were chosen as the reference. The individual spot intensity volume was normalized using the total intensity volume (sum of the intensity volumes obtained from all spots in the same 2-D electrophoresis gel). The normalized intensity volume values of individual protein spots were then used to determine the differential protein expression between stages. 2-D spots that exhibited a 1.5-fold or more decrease or increase were excised for identification by MALDI-TOF mass spectrometry.

Protein digestion and identification

Spots were excised from the 2-D gels and subjected to overnight incubation with sequencing-grade trypsin, as described by Westermeier and Naven (2004). The samples were desalted using Perfect Pure C-18 tips (Eppendorf) according to the manufacturer protocol, and the peptides were eluted using 1 μL of a freshly prepared 10 g/L solution of 4-hydroxy-α-cyanocinnamic acid in 0.1% TFA (v/v) and 50% methanol (v/v). This sample was applied onto a MALDI-TOF target and allowed to dry. Mass spectra in the m/z range of 800 to 3200 were acquired using a MALDI-TOF mass spectrometer (Bruker Daltonics) operating in positive reflection mode and pre-calibrated with a mixture of mass standards. Monoisotopic mass peaks lists were created using Flex Analysis 3.0 (Bruker Daltonics). When trypsin autolysis peaks were identified, they were used as internal mass calibrants. Protein identification by peptide mass fingerprinting was performed using the PiumsGUI software (Samuelsson et al., 2004) running locally, and multiple MS1 searches were made automatically. In the search parameters, carbamidomethylation was set as a fixed modification and methionine oxidation as a variable one, one missed cleavage was allowed, and the monoisotopic mass error was less

than 100 ppm. The searches were performed against *C. arabica* and *C. canephora* EST-based protein databases that were obtained from the HarvEST search engine software (http://harvest. ucr.edu/). For positive match, we used the search criteria of at least five peptides showing matches with a minimal score of 5.0 and P-value below 0.05. Additionally, positive protein assignments required greater than 15% sequence coverage and less than 25% deviation between theoretical and experimental MW and pI values obtained from calibrated 2-D gels. NCBI-BLAST (http://www.ncbi.nlm.nih.gov) was used to find the known function of a protein for the corresponding sequences obtained from PIUMS.

RESULTS AND DISCUSSION

Comparative analysis of proteomic maps

In this study, 2-D gels of the two different fruit developmental stages (stage 1, at 55 DAA; and stage 2, at 90 DAA) were compared using immobilized gradient strips with a pH range of 3 to 10. All 2-D gels from each condition assessed were analyzed using the same parameters of hue, contrast, and sharpness. Similar profiles were found between stage 1 (Figure 1A) and stage 2 (Figure 1B), with approximately 300 well-resolved polypeptide spots distributed throughout the gel. Most of these spots ranged in mass from 22 to 66 kDa and in pI from 4.0 to 7.0. Proteomic analysis of later coffee seed maturation stages (210 and 255 DAA) showed a profile with approximately 185 spots, mostly with pI varying from 5.0 to 7.0 and mass from 10 to 115 kDa (Franco et al., 2009). The variation in the number of expressed proteins in the early and later fruit developmental stages reflects the characteristic metabolism of each phase. In the early developmental stages of *C. arabica* fruit, the perisperm is the site of biosynthesis of several important biochemical precursors that are then loaded, transformed, and stored in the mature endosperm. In the same way, the intense accumulation of storage compounds during endosperm development is preceded by import of amino acids and metabolites, such as sugars, in the perisperm (Geromel et al., 2006; Salmona et al., 2008; Joët et al., 2009). After the comparison between the reference gels of the two analyzed stages, statistical analysis pointed out six protein spots that were differentially expressed (Table 1 and Figure 1A and B).
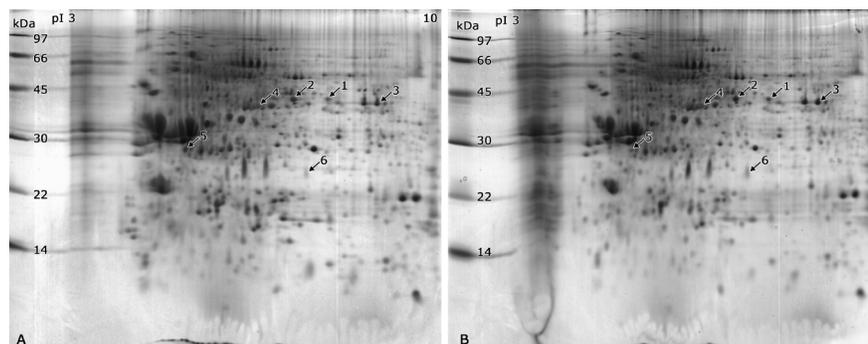
Figure 1. Two-dimensional electrophoresis images of coffee fruits proteins expressed in stage 1 (55 DAA) (**A**) and stage 2 of development (90 DAA) (**B**). Total extract proteins separated by a strip of 11 cm with a pH gradient 3-10, followed by a vertical electrophoresis SDS-PAGE 11.7% acrylamide (18 x 18 cm) and subsequently stained with Coomassie blue.

Table 1. Statistical analysis of differentially expressed protein spots between stages 1 and 2.

Spot[a]	Relative expression of the spot[b]	Mean (100%)[c]	M.S.D.[d]	%Vol stage 1[e]	%Vol stage 2[f]	t-test[g]
1	1.53074	0.496149	0.1128	0.383736	0.608562	17.3273
2	1.50008	0.238891	0.0801	0.164603	0.31318	3.50975
3	1.71979	0.521646	0.1458	0.37608	0.667213	25.0036
4	1.50811	0.26405	0.0571	0.207099	0.321002	23.7493
5	1.56565	0.29728	0.0948	0.385811	0.208749	3.6955
6	1.84539	0.122433	0.0531	0.0717643	0.173101	4.48462

[a]Spot number. [b]Ratio value inter-class analysis, expressed as volume percentage, indicates that the spots are at least 1.5 times higher or lower in one of the stages than the other one. [c]Sum of all the sample values divided by the sample size. [d]Mean squared deviation, the square root of the average squared difference of each sample value to the center location. [e]Center value of spots from stage 1 expressed as volume percentage. [f]Center value of spots from stage 2 expressed as volume percentage. [g]Two-sample t-test indicates that the difference between the two stages is statistically significant when this value is larger than a certain threshold (Westermeier and Naven, 2004). The threshold chosen was 1.5.

Mass spectrometry protein identification

The differentially expressed protein spots were subjected to trypsin digestion and MALDI-TOF analysis and were then identified (Table 2).

Table 2. Proteins identified.

Protein[a]	Spot No.[b]	NCBI accession No.[c]	Species[d]	Score[e]	pI[f]	MW[g]	P value[h]	Identical masses[i]	Coverage[j]
Fructose bisphosphate aldolase	1	P29356.1	*Spinacia oleracea*	39.99	7.8 (8.3)	44 (38)	0	11/27	48.1
Fructose bisphosphate aldolase	2	P29356.1	*Spinacia oleracea*	52.46	7.2 (6.9)	43 (38)	0	16/51	52.7
Glyceraldehyde-3-phosphate dehydrogenase	3	P25861.1	*Antirrhinum majus*	43.01	8.7 (8.5)	42 (36)	0	13/35	56.4
Cytosolic malate dehydrogenase	4	Q08062.2	*Zea mays*	28.32	6.5 (6.3)	40 (35)	0	9/39	35.6
Thaumatin	5	P13046.1	*Nicotiana tabacum*	21.05	5.2 (5.2)	29 (24)	0.005	5/32	25.4
Protein 11S	6	P13744.1	*Cucurbita maxima*	23.07	7.4 (6.5)	26 (54)	0.011	7/21	14.1

Peptides mass fingerprint (PMF) allowed the identification of corresponding proteins by using PIUMS and *Coffea* ESTs data. [a]Identified protein based on MS analysis of spots excised from fruit coffee 2-D gels. [b]Spot numbers correlated with those from Figure 1. [c]NCBI accession No. of the respective species. [d]Species that showed the highest score in blast analysis after coffee EST submission. [e]Probability that the observed match between the experimental data and the database sequence is a random event, expressed as -10*Log(P). [f]Observed isoelectric point (theoretical). [g]Observed molecular weight (theoretical). [h]Statistical probability that the observed degree of matching would be found by chance. [i]Number of matched/searched peaks. [j]Percentage of sequence coverage calculated by dividing the number of amino acids observed by the protein amino acid length.

Protein spots 1/2, 3, and 4, detected in higher amount in stage 2 (Table 1), were identified as fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, and cytosolic malate dehydrogenase, respectively (Table 2). The enzymes fructose bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase have also been identified in proteomic studies of apple, strawberry (Zheng et al., 2007), and tomato (Faurobert et al., 2007). Malate dehydrogenase has been identified in citrus studies (Katz et al., 2007). Despite the fact that both enzymes are involved in carbohydrate anabolism and catabolism, their higher expression in stage 2 suggests activation of the glycolytic pathway.

Stage 2 represents the perisperm-endosperm early transition. The early development of the coffee seed (transient perisperm) is characterized by active starch biosynthesis, with a

high proportion of glucose derived from sucrose catabolism, the major carbohydrate transported in the phloem of coffee trees (Geromel et al., 2006; Joët et al., 2009). When the endosperm started expanding, the glucose content dropped and lipids and chlorogenic acids started to accumulate. In this stage, the glucose is metabolized by glycolysis and utilized as the carbon skeleton source, and provides energy for lipids and chlorogenic acids syntheses (Geromel et al., 2006; Lepelley et al., 2007; Joët et al., 2009; Troncoso-Ponce et al., 2009).

Two different spots (1 and 2) were identified as fructose bisphosphate aldolase, suggesting the presence of a posttranslational modification event that changed the observed pI from 7.2 to 7.8 without significant changes in the protein molecular weight. Considering that plant glycolysis exists in both the cytosol and the plastid, and the parallel reactions are catalyzed by distinct nuclear-encoded isozymes (Plaxton, 1996), it is possible that these enzymes occupy distinct cell compartments.

The protein spot 5, present in higher concentration in stage 1 (Table 1), was identified as thaumatin (Table 2) and may also be related to sugar metabolism. Thaumatin belongs to the thaumatin-like protein (TLP) family, which includes the sweet-tasting protein extracted from *Thaumatococcus daniellii*, a plant native to West Africa. This protein is on average 100,000 times sweeter than sugar (de Vos et al., 1985). The expression of TLPs is related to some stressors, such as osmotic stress in tomato (Rodrigo et al., 1991), salinity in maize (Frendo et al., 1992), and pathogen attack in beans (Ye et al., 1999). Analyses have revealed that one of the many genes induced by drought in carrot is a member of the thaumatin family (Jung et al., 2005). Pasternak et al. (2007) showed that oxidative stress-inducing agents might promote cell division in plant, a process that occurs intensively in this perisperm elongation phase (Pezzopane et al., 2003; De Castro and Marraccini, 2006). Therefore, the identification of thaumatin indicates that the coffee seed in the early developmental stage is under some stress condition. However, additional studies are necessary to identify the stress origin in stage 1.

The protein spot 6, detected in higher concentrations in stage 2 (Table 1), was identified as the protein 11S (Table 2). The molecular and biochemical characterizations of the 11S storage protein were performed by Rogers et al. (1999a). This protein is the major source of peptides and amino acids in the fruit endosperm. The accumulation of these proteins occurs throughout fruit development, and peaks in the first half of the period of fruit ripening, which corresponds to the phase of maximum expansion of endosperm grains (approximately 14 weeks after flowering) (Rogers et al., 1999a). These proteins have a relatively low content of the sulfur amino acids cysteine (1%) and methionine (0.6%), suggesting a limited role of these amino acids as precursors of aromas and flavors in coffee (Grosch, 2001).

In summary, six differentially expressed protein spots were identified by mass spectrometry. The majority of them are involved directly or indirectly in the carbohydrate metabolism of the immature fruit of *C. arabica*, possibly due to the requirement of immediate energy for development. This proteomic study contributes to a better understanding of the early developmental stage of the coffee fruit, and shows that during the perisperm elongation and the early perisperm replacement by endosperm, there are only a few metabolic changes detectable by the 2-D electrophoresis technique.

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