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Endo-β-mannanase and β-tubulin gene expression during the final phases of coffee seed maturation

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ABSTRACT. Coffee seeds begin to develop shortly after fertilization and can take 6 to 8 months to complete their formation, a period during which all the characteristics of the mature seed are determined, directly influencing physiological quality. However, little is known about the molecular mechanisms that act during coffee seed maturation. The objective of the current study was to analyze expression of the β-tubulin *(TUB*) and endo-β-mannanase (*MAN*) genes during different phases at the end of development and in different tissues of *Coffea arabica* seeds. The transcription levels of the *TUB* and *MAN* genes were quantified in a relative manner using qRT-PCR in whole seeds, and dissected into embryos and endosperms at different developmental stages. Greater expression of *MAN* was observed in whole seeds and in endosperms during the green stage, and in the embryo during the over-ripe stage. High *TUB* gene expression was observed in whole seeds during the green stage and, in the embryos, there were peaks in expression during

the over-ripe stage. In endosperms, the peak of expression occurred in both the green stage and in the cherry stage. These results suggest participation of endo-*β*-mannanase during the initial seed developmental stages, and in the stages of physiological maturity in the embryo tissues. *TUB* gene expression varied depending on the developmental stage and section of seed analyzed, indicating the participation of *β*-tubulin during organogenesis and coffee seed maturation.

Key words: qRT-PCR; *Coffea arabica*; Developmental stages

INTRODUCTION

Coffee is considered a very important commodity in the world market and is produced and consumed in various countries. Because of its economic importance, several studies have been carried out in order to develop new technologies to increase production and improve coffee quality. However, little is known about the molecular mechanisms that act during coffee seed formation when all the characteristics of the mature seed are determined, which have a direct influence on seedling quality and establishment of the crop. Coffee seeds develop shortly after fertilization, and complete seed formation can take 6 to 8 months in the case of arabica coffee (de Castro and Marraccini, 2006). During this period, several metabolic processes are responsible for seed formation and the development of characteristics related to final seed quality.

The different development and maturation stages of coffee seeds are currently identified using a phenological scale based on visual aspects of the berry. Five maturation stages are suggested, classified as green, yellow-green, cherry, over-ripe, and dry (Pezzopane et al., 2003). Several genes that perform functions in numerous physiological processes and in embryonic development and maturation have been characterized in coffee. Genes have recently been identified as having differential levels of expression associated with the five maturation stages. For example, α-galactosidase can be used as a marker of the green stage, caffeine synthase can be used as a marker of the transition from the green to the yellow-green stage, and isocitrate lyase and ethylene receptor 3 can be used as markers of the final stages of seed maturation (Gaspari-Pezzopane et al., 2012).

The enzymes α-galactosidase, *β*-mannosidase, and endo-*β*-mannanase are normally considered directly responsible for galactomannan hydrolysis during seed germination (Grant Reid and Meier, 1973; Marraccini et al., 2000). Also during seed germination, endo-*β*-mannanase stores cell wall polysaccharides, such as mannans, galactomannans, and glucomannans. Previously studies have concentrated on the role of endo-*β*-mannanase in softening the cell wall found close to the embryo to allow root emergence (Nonogaki et al., 2000; da Silva et al., 2004; 2008).

However, other genes have potential for use as molecular markers in breeding programs, as is the case of the gene that encodes *β*-tubulin, a protein component of the microtubules, which are essential for cell expansion and division processes (Goddard et al., 1994; de Castro and Marraccini, 2006). Using western blot analysis, different *β*-tubulin accumulation profiles have been observed, along with different profiles of the microtubular cytoskeleton in the different tissues and development times. A sequential pattern of decreasing protein accumulation with increasing development was observed until the protein reached undetectable levels in the final maturation stages (Estanislau, 2002; de Castro and Marraccini, 2006).

Furthermore, renewed production of this protein was observed during coffee seed imbibition, which is part of the coffee seed germination process (da Silva et al., 2008).

In contrast, genes usually associated with seed germination can also act during seed maturation. Endo-*β*-mannanase is known to be an essential enzyme in coffee seed germination, and high activity rates have been observed during coffee seed imbibition and germination. In other species, studies have detected different encoding genes of this enzyme that characterize aspects of changes in gene expression on both the spatial and temporal levels in the seeds. In tomato, different genes have been identified that encode endo-*β*-mannanase involved in different functions. The *LeMAN2* gene is associated with breaking the endosperm and enabling root emergence in seeds (Nonogaki et al., 2000); the *LeMAN1* gene is related to mobilizing reserves shortly after seed germination (Bewley et al., 1997); *LeMAN3* and *LeMAN4* are expressed during berry maturation (Bewley et al., 2000; Banik et al., 2001); and the *LeMAN5* gene has been isolated from tomato anthers and pollen (Filichkin et al., 2004).

Furthermore, different spatial and temporal expression patterns have been observed for the *DcMAN1* gene and endo-*β*-mannanase enzyme activity in carrot seeds with immature embryos, suggesting that this gene is associated with finalization of the development process in carrot seeds when imbibed (Homrichhausen et al., 2003).

In coffee seeds, different endo-*β*-mannanase isoforms have been reported. According to da Silva et al. (2004), there are four different isoforms in coffee seed endosperms, while Marraccini et al. (2001) observed eight. This difference may be due to the researchers having used different materials; the first used imbibed seeds and the second worked with germinated seeds. These results suggest that the different isoforms of the endo-*β*-mannanase enzyme have different functions during coffee seed germination and subsequent seedling growth. However, little is known about the expression of these genes during development and maturation and whether they could be used as event markers during coffee seed formation. Thus, the objective of the present study was to investigate expression of the *β*-tubulin (*TUB*) and endo-*β*-mannanase (*MAN*) genes at different developmental stages and in different coffee seed tissues (*Coffea arabica*).

MATERIAL AND METHODS

Coffee berries from the Rubi cultivar were picked during the green, yellow-green, cherry, over-ripe, and dry developmental stages in an experimental field at Universidade Federal de Lavras (UFLA, Federal University of Lavras), Lavras, MG, Brazil. Each stage was obtained at a different time, typically when most of the berries of the plants were at the stage that was the object of the harvest. The berries were picked from the middle branches of the plants and the central parts of these branches in randomly selected plants. After being harvested, the berries from each stage were selected to standardize the maturity stage, using the phenological scale proposed by Pezzopane et al. (2003). The moisture content of the berries from each stage was determined using the drying chamber method (105 ± 3°C for 24 h) (Brasil, 2009).

The seeds were separated from the berries by hand using a utility knife, and the following plant materials were extracted: embryos, endosperms, and whole seeds. Half the extracted plant materials were frozen in liquid nitrogen and stored at -80°C and the other half was used in the germination test.

The germination test was carried out with four 50-seed replicates for each phenologi-

cal stage. The seeds were placed on germination paper, moistened with a quantity of water equivalent to 2½ times the weight of the dry substrate, and maintained in a germinator at 30°C in light. Seeds were assessed 15 and 30 days after sowing according to the RAS (Brasil, 2009) and the results were expressed as a percentage. At 15 days after sowing, the percentage of root protrusion and normal seedlings were assessed. Protrusion was considered as visual identification of the white-colored embryonic axis, and a normal seedling presented visual morphological differentiation of the main root and at least two secondary roots. At 30 days after sowing, the percentage of normal and normal-strong seedlings was assessed. A normal-strong seedling was considered as having a root longer than 2.5 cm and the presence of at least two secondary roots. At 45 days after sowing, the seedlings with open cotyledon leaves were counted and the results expressed as a percentage.

Gene expression

All materials used to extract RNA were treated with diethylpyrocarbonate at 0.5% to inactivate RNases. All solutions used were prepared with RNase-free autoclaved distilled water. Plant RNA Purification Reagent (Invitrogen) was used to extract RNA according to the manufacturer protocol; 500 µg chilled reagent (4°C) was added to each microtubule containing approximately 100 µg of macerated tissue and homogenized in a vortex. The tubes were then incubated for 5 min at ambient temperature and left in a horizontal position to maximize RNA extraction. After this period, the material was centrifuged for 2 min at ambient temperature at 12,000 *g* and the supernatant was transferred to a new tube with 100 µL 5 M NaCl and homogenized in a vortex. Then, 300 µL chloroform was added and samples were homogenized by inversion. To separate the phases, the samples were centrifuged for 10 min at 4°C (12,000 *g*) and the upper aqueous phase was transferred to a new tube. Next, a volume equivalent to the aqueous phase of chilled isopropanol (approximately 400 µL) was added and shaken in a vortex for 5 s. The samples were maintained at ambient temperature for 10 min and then centrifuged for 10 min at 4°C (12,000 *g*). The supernatant was discarded, the pellet washed in 1 mL chilled 75% ethanol, and the tubes were centrifuged for 1 min at ambient temperature (12,000 *g*). The residual liquid was removed from the tube with a pipette and the RNA was re-suspended in 20 µL RNAse-free water. The samples were stored at -20°C.

The quantity and quality of total RNA was assessed using a Nanovue Plus spectrophotometer (Healthcare, Piscataway, NJ, USA). To assess the integrity of the extracted samples, the RNA was assessed on a 1.5% agarose gel stained with ethidium bromide and viewed under ultraviolet light. The image was captured using the EDAS 290 (Kodak®) photodocumenter. The complete RNA was treated with DNase I (RNase Free) (Ambion).

The RNA was purified using the RNeasy MinElute Cleanup kit (QIAGEN). This purification step was carried out to ensure the removal of genomic DNA and salts resulting from the extraction, which have been proven to damage PCR efficiency. A conventional PCR was carried out, followed by observation on a 1.5% agarose gel to detect the possible presence of genomic DNA contaminating the material.

The HighCapacity cDNA Reverse Transcription (Applied Biosystems) kit was used to synthesize cDNA. First, the RNA was prepared at a concentration of 1 µg in a final volume of 10 µL. A mixture was then prepared containing 2 µL 10X enzyme buffer, 2 µL 10X RT Random Primers, 0.8 µL dNTP mix (100 mM), 1 µL MultiScribeTM Reverse Transcriptase, and water, for a final volume of 10 µL. Ten µL of this mixture was added to each prepared 10

µL 1 µg RNA solution. The tubes were placed in a thermocycler programmed with three steps: 10 min at 25°C to anneal the primers, 2 h at 37°C for the enzyme to act, and 5 min at 85°C to inactivate the enzyme. The samples were stored at -20°C.

The cDNA was used as a template to analyze qualitative gene expression in an ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) using SYBR Green. Little is known about the genes responsible for the coffee seed maturation process, so EST searches were conducted with previously identified *C. arabica* sequences and sequences from other species placed in the NCBI (National Center for Biotechnology Information). The sequences were obtained from a contig sequence bank of the Coffee Genome Project. The primers were designed using the PrimerExpress 4.1 software, and are shown in Table 1.

Table 1. Specific primers used in real-time PCR analysis of *Coffea arabica* seeds.

Gene	Forward (5' → 3')	Reverse (5' → 3')
MAN	CACAATCATGGCATGGGAACT	ATTGACTGTCCTTCCGGAGTAATC
TUB	CCAGAGCTCACTCAGCAAATGT	GCGGATCAGCTGCACACA
UBQ	CGCTGACTACAATATCCAAAAGGA	CTGCATTCCACCCCTCAGA
14-3-3 protein	TGTGCTCTTTAGCTTCCAAACG	CTTCACGAGACATATTGTCTTACTCAAA

First, an absolute quantification trial was used where the standard curve, primer efficiency, and the best solution for the samples were determined. A serial dilution of cDNA was prepared at 1:5, 1:25, 1:125, 1:625, and 1:325.

After determining that a 1:5 dilution was appropriate and primer efficiency was 94-97%, relative expression trials were carried out using the comparative CT method with three replicates for each sample, including negative and endogenous controls. The expression of genes encoding endo-*β*-mannanase and *β*-tubulin was analyzed (*MAN* and *TUB* genes). The thermal conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, and finalized with 1 s at 95°C. The data were analyzed using the software v.2.0.1 of the ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) in the Central Seed Laboratory /UFLA. The reaction conditions used were 2 µL cDNA, 10 µM of each primer, and 7.5 µL SYBR® Green PCR Master Mix (Applied Biosystems) for a final volume of 15 µL/sample.

To calculate gene expression, each sample was first normalized with the endogenous controls 14-3-3 and ubiquitin, and relative quantification was obtained using the following equations:

$$\Delta Ct = Ct_{(TG)} - Ct_{(MRG)}$$
$$\Delta\Delta Ct = \Delta Ct - \Delta Ct_{(Control)}$$
$$\text{Relative quantification} = 2^{-\Delta\Delta Ct} \quad \text{(Equation 1)}$$

where, *Ct* is cycle threshold, *TG* is target gene, and *MRG* is mean of the reference gene. The threshold was defined automatically. The data are reported as mean values ± SD. Significant differences ($P < 0.005$) between mean values were determined for each variable using the Scott Knott test to rank the experimental treatments.

RESULTS

Greater physiological potential was observed in seeds picked during the cherry and over-ripe stages (Table 2). Seeds picked during the green stage had low germination and vigor

values, and the seeds picked during the yellow-green and dry stages germinated, but with poor results. Decreased moisture content was observed in the seeds throughout their development, especially in the last two developmental stages (Table 2).

The gene expression data of the *MAN* transcript in different sections of the seeds and during different developmental stages are shown in Figure 1. Expression patterns differed in the various seed parts and during the different developmental stages. A peak in transcription was observed in the gene linked to endo-*β*-mannanase (*MAN*) synthesis in whole seeds during the green stage, followed by the yellow-green stage. During the remaining developmental stages, low relative expression of this gene was observed.

Upon analyzing expression of the *MAN* gene in the separate seed parts, it may be seen that gene expression peaked in embryos extracted from coffee seeds during the over-ripe stage but expression levels were lower during the other developmental stages. In the endosperm, higher expression levels were observed in the early developmental stages.

Expression of the *TUB* gene linked to *β*-tubulin synthesis also differed among the different seed parts and during the different developmental stages (Figure 2). In whole seeds, higher expression of the *TUB* gene was observed during the green stage compared to the other developmental stages. Analysis of the separate seed parts (embryo and endosperm) showed that in the embryo, there were peaks in expression of the *TUB* gene during the over-ripe stage, followed by the green and dry stages. There was no difference in expression of the *TUB* gene between the yellow-green and cherry stages when analyzed in the embryo. In the endosperm, a peak in expression was observed during the green and cherry stages, with lower expression levels during the other developmental stages (Figure 2).

Table 2. Water content, mean percentage of root protrusion, normal seedlings, normal strong seedlings, and open cotyledon leaves of *Coffea arabica* seeds obtained from berries collected during different development stages.

Maturation stage	Water content (%)	Radical protrusion (%)	Normal seedlings (%)	Normal strong seedlings (%)	Opened Cotyledon (%)
Green	69.94	1.00D	1.00D	1.00D	0.00E
Yellow green	66.28	42.00C	76.00B	63.33B	32.33C
Cherry	68.51	89.00A	82.87A	88.00A	58.34A
Over-ripe	49.90	89.67A	86.75A	82.33A	42.33B
Dry	23.06	66.34B	27.00C	10.34C	11.34D

Means followed by the same letter in the columns do not differ significantly at the 5% level using the Scott-Knot test.

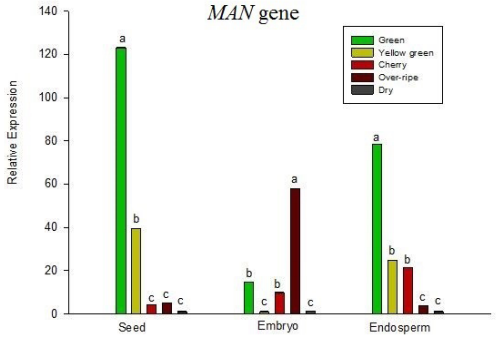

Figure 1. Quantification of *MAN* gene expression in *Coffea arabica* seeds during the green, yellow-green, cherry, over-ripe, and dry stages in different seed parts (whole seeds, embryos, and endosperms).

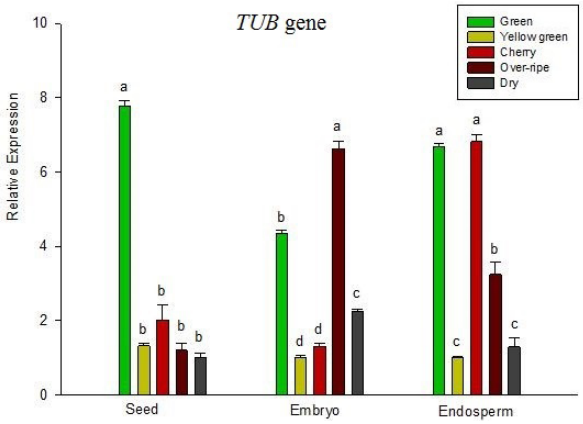

Figure 2. Quantification of *TUB* gene expression in *Coffea arabica* seeds during the green, yellow-green, cherry, over-ripe, and dry stages in different seed parts (whole seeds, embryos, and endosperms).

DISCUSSION

The cherry stage is typically used for seedling production and experiments, and it is understood that the seeds during this stage are at the point of physiological maturity, exhibiting maximum physiological quality. However, the results of the present study reveal that both the cherry and the over-ripe stages have similar physiological qualities (Table 2).

Seed maturation involves regulatory networks that integrate genetic programming and metabolic and hormonal signals to induce compound accumulation for storage, reduce moisture content, increase ABA levels, and establish drought tolerance and primary dormancy (Holdsworth et al., 2008).

Initiating from specific gene signaling, complete maturity can occur, determining the germination ability and seed vigor. The *MAN* gene is known to be involved in seed germination of various species in addition to coffee seeds (Mo and Bewley, 2003; Buckeridge et al., 2004; da Silva et al., 2004). The results of the present study also show expression of this gene in the first developmental stage (green), suggesting participation of this gene in other processes within the seed. The *MAN* transcript peak during the green stage of seed development can be explained by the high metabolic activity that happens at this stage for production of reserve compounds. Plants show different strategies, including accumulation of large quantities of substances, that are mobilized during development, and their products are used for various purposes, such as energy generation and raw material production (proteins, nucleic acids, carbohydrates, and fat) for cell and tissue construction (Buckeridge et al., 2000). In contrast, at more advanced stages, such as during the cherry and over-ripe stages, endo-β-mannanase is required to break the cell wall, especially in the cap region where the endosperm ruptures for the embryo to grow. At the same time, reserve compounds are broken down to supply energy necessary for embryo development at the time of germination. The cell wall is an extremely dynamic structure that can be modified during the different developmental stages of the plant, thus allowing various physiological events to occur, such as seed germination, development through cell expansion, leaf and flower abscission, and fruit ripening (Campbell and Braam, 1999). In the case of coffee seeds, the embryo is completely different, even before germination (da Silva et al., 2004)

Analyses of the proteome in developing coffee berries showed the presence of enzymes, such as fructose biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, and malate dehydrogenase, which are involved in carbohydrate metabolism (Bandil, 2008). According to Bandil (2008), the berry is at a stage of cell division and expansion and needs energy to ensure its growth. Considering that coffee seeds in the green stage are still at the developmental stage, it is suggested that endo-*β*-mannanase is required at this stage to produce energy to continue the maturation process. Figure 1 shows that generally as the developmental stages advance, endo-*β*-mannanase enzyme transcription decreases. In contrast, Gaspari-Pezzopane et al. (2012) observed transcript accumulation of genes associated with carbohydrate synthesis (*GAL* and *MAN-B*), and aromatic components were observed in the final developmental stages. It should be noted that in the present study the seeds were not pre-imbibed, unlike other studies that used pre-imbibed seeds during the cherry stage.

According to da Silva et al. (2004), in imbibed seeds, endo-*β*-mannanase activity occurs first in the "cap" endosperm (part of the endosperm close to the embryo) and only later in the rest of the endosperm. In the present study, greater expression of the gene that encodes endo-*β*-mannanase was observed in embryos extracted from coffee seeds during the over-ripe stage. These results suggest that even without imbibition, possible synthesis of the endo-*β*-mannanase enzyme occurs during more advanced stages, preparing the seeds for a possible germination process. Especially in coffee seeds (*C. arabica*) that are considered intermediary, there is a whole mechanism of seed preparation for germination shortly after maturity, avoiding natural desiccation processes (Hong and Ellis, 1996; Gentil, 2001; Rosa et al., 2006).

Microtubules are essential structures of the cell skeleton or cytoskeleton and are considered the framework that determines the form and distribution of the cell constituents. In the present study, it was seen that expression of the *β*-tubulin gene in whole seeds was lower than in the endosperm and embryo. According to José et al. (2009), since the selected genes are expected to be expressed exclusively in the embryo, using whole seeds for RNA extraction may have resulted in considerable dilution of the embryonic RNA. The embryo volume is estimated as 1/40 of the whole coffee seed.

The *β*-tubulins are the main proteins that form cylindrical protein filaments called microtubules that, in turn, are responsible for the formation of the eukaryote cytoskeleton (Stotz and Long, 1999). In plants, the microtubules control important growth and morphogenesis processes and take part in responses to biotic and abiotic stress (Morello et al., 2002). The same authors stated that the regulation of expression of the *β*-tubulin gene was complex and involved mechanisms with various phases.

According to de Castro and Marraccini (2006), from 90 to 150 days after fertilization (DAF), the perisperm cells widen, histodifferentiation occurs, and the endosperm and embryo grow, and as of this point, intense mitosis and expansion activities can be observed. At 150 DAF, organogenesis resumes in order to complete maturation and there is complete degradation of the microtubule network and a decrease in the *β*-tubulin content in all tissues at the final maturation stage. This is shown in Figure 2, where there were high expression levels during the green stage, characterizing the organogenesis process and this expression decreased during the final developmental stage, especially when only the embryo was analyzed. This decrease in *TUB* expression at the end of maturation is characteristic in seeds once maturation is complete (de Castro, 1998; Jing et al., 1999; de Castro et al., 2000, 2005).

In conclusion, the *MAN* gene is expressed during the early stages of developing seeds and embryos. *TUB* gene expression varied depending on the developmental stage and the sec-

tion of the seed being analyzed, and indicated the involvement of *β*-tubulin during organogenesis and maturation of coffee seeds.

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