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GA_3 and ABI_3 gene expression in coffee seeds during maturation

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ABSTRACT. During maturation in seeds, metabolic processes dependent on gene expression are controlled by hormones, including abscisic acid and gibberellin. These hormones determine dormancy or the capacity for germination in seeds and may have different expression levels in the endosperm and embryo of coffee seeds. We quantified gene expression in the biosynthetic pathway of gibberellin and abscisic acid in different parts of *Coffea arabica* L. seeds in pre and post physiological maturity, in order to better understand the germination mechanisms of this species. Coffee fruits were harvested at green, yellowish-green, cherry, over-ripe and dry stages. For studies of gene expression, intact seeds were used in addition to endosperm and isolated embryos. The RNA from different tissues was extracted and treated with DNAse to synthesize cDNA. Transition levels of $CaGA_3$ (gibberellin) and $CaABI_3$ (abscisic acid) genes were quantified with qRT-PCR, using specific primers for coffee. Intact seeds at each phenological stage were submitted to germination tests, to evaluate the physiological quality of the seeds. Seed harvested at green and dry stages showed lower physiological

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quality when compared to the other maturation stages, and germination at the green stage was close to zero. Greatest expression of $CaGA_3$ and $CaABI_3$ occurs in the endosperm of coffee seeds in cherry and over-ripe stages, with better physiological performance. There are differences in $CaABI_3$ and $CaGA_3$ gene expression in embryos and endosperm of the coffee seeds.

Key words: *Coffea arabica*; Gibberellic acid; Abscisic acid; qRT-PCR

INTRODUCTION

Coffee is an important product of the Brazilian trade balance, with Brazil being the world's largest producer and exporter of this commodity. In 2018 the country produced approximately 45 million bags (National Company Of Suppy - Conab, 2019), with Minas Gerais being the main producer state, with 54% of national production (Conab, 2019). Due to it's economic importance, research using molecular markers has been conducted to better understand metabolic reactions involved in the maturation of coffee seeds (José et al., 2009, Venglat et al., 2011).

Coffea arabica L. seeds complete their development from six to eight months after fertilization (De Castro & Marraccini, 2006). During this time, several metabolic processes take part in succession to impart specific characteristics such as reserve accumulation, germination, dormancy, desiccation tolerance, among others. Such processes rely on the expression of specific genes at each stage of seed formation and are most often controlled by plant hormones.

The process of germination of coffee seeds is not yet fully elucidated, mainly due to the characteristic of slow germination of the specie. Germination depends on the embryo's ability to reactivate its metabolic activity (Rajjou et al., 2012). Achard et al. (2009) and Magome et al. (2008) found that germination is under strict regulation of plant hormones, including gibberellin (GA), abscisic acid (ABA), auxin and ethylene (Han and Yang, 2015). Germination is also significantly affected by several environmental factors, such as various abiotic stresses (Rajjou et al., 2012; Han and Yang, 2015). These factors mainly affect metabolism and the different signaling pathways of GA and ABA (Holdsworth et al., 2008).

Abscisic acid (ABA) is a plant hormone related to the accumulation of reserve components, acquisition of desiccation tolerance, induction of primary dormancy and suppression of early germination (Kanno et al., 2010). In coffee seeds it is associated with inhibition of endosperm weakening (Silva et al., 2004, Müller et al., 2006), which may lead to a germination delay. Silva et al. (2008) observed a transient increase in endogenous ABA content in embryo cells during germination of coffee seeds, which inhibit the extensibility of the cell wall, restricting turgor increase, together with inhibition of endo-β-mannanase enzyme activity contributing to delay the germination process.

On the other hand, gibberellin (GA_3) can contribute to the germination process and is essential during embryogenesis. The production of this hormone in the embryo and/or endosperm during seed development is required for formation of normal seeds (Singh et al., 2002). During soaking, the gibberellins promote endosperm weakening, which seems to be a widespread phenomenon (Finch-Savage and Leubner-Metzger, 2006), suggesting that

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GA_3 regulates molecular mechanisms related to endosperm weakening, at least in part. In coffee seeds GA_3 de novo biosynthesis is required for the elongation of embryo cells and endosperm breakage during germination (Silva et al. 2005). The levels of ABA and GA in seeds are what determine dormancy or the capacity for germination (Clemente et al., 2015). According to Schuch et al. (1990), coffee fruits undergo cell division 6-8 weeks after fertilization, but grow little in weight or volume during that period. High levels of ABA and low levels of GA in this period is believed to be a form of true dormancy of fruit growth.

The endosperm seems to be the main barrier responsible for preventing or delaying the coffee seed germination. It has been observed that cotyledon embryos not fully differentiated and developed when isolated from seeds with 120 and 150 days after anthesis (DAA), are capable of germinating and developing normal seedlings when incubated in culture medium, demonstrating that the embryos acquire the ability to germinate yet immature (Estanislau, 2002). Moreover, intact seeds are capable of germinating only in the later stages of maturation, around 225 DAA. This indicates a repression mechanism of coffee seed germination in early stages of development (Eira et al., 2006). This mechanism can be controlled by several factors, such as the balance of abscisic acid and gibberellins levels, as observed in other species, maintaining both embryo and seed in the development mode (Eira et al., 2006, Hermann et al., 2007; Kanno et al., 2010; Clemente, et al., 2013).

Although coffee quality is largely dependent on fruit development and final grain composition, few studies on the genetic and physiological maturation control are available for coffee seeds. Furthermore, the role of hormones like ABA and GA_3 in coffee seed germination has been studied, but little is known about these syntheses during seed development in this species. Thus, it has been proposed to quantify gene expression in the biosynthetic route of gibberellin and abscisic acid in different parts of *C. arabica* seeds in final stages of maturation, in order to better understand the germination mechanisms of the species.

MATERIAL AND METHODS

Biological material and processing of fruits

Coffea arabica fruits, Rubi cultivar were harvested in the experimental field of Universidade Federal de Lavras (UFLA) at five maturation stages, green, yellowish-green, cherry, over-ripe and dry. Each stage was obtained in a different time in random plants.

Fruits were harvested from middle plant branches and from the medial parts of these branches. After harvest, fruits of each stage were selected for maturation uniformity, considering the phenological scale proposed by Gaspari-Pezzopane et al. (2012), and were subjected to the determination of water content by oven method (105°C for 24 h) (Brasil, 2009).

The seeds were separated from the berries by hand using a utility scapel, and the following plant materials were extracted: embryos, endosperms, and whole seeds. This activity was carried out with due care so that the scalpel did not cut the 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 moisture content of the berries of each stage was determined using the drying chamber method (105ºC for 24 h) (Brasil, 2009).

Physiological analyzes

Intact seeds of each phenological stage were submitted to physiological quality evaluation, through the germination test, conducted with four replications of 50 seeds. Seeds were distributed in a germination paper, moistened with water equivalent to two and a half times the weight amount of dry substrate and were maintained in a germination chamber at a temperature of 30°C in the presence of light. The evaluations were performed at fifteen and thirty days after sowing, according to criteria from the Rules for Seed Analysis (Brasil, 2009) and the results expressed as percentage. At 15 days after sowing, the percentage of root protrusion and normal seedlings was evaluated. It was considered as protrusion the visual identification of the embryonic axis, with white color and as normal seedling the ones that visually showed the morphological differentiation of the main root together with at least two sary roots. At 30 days after sowing, it was evaluated the percentage of normal seedlings and strong normal seedlings. It was considered as a strong normal seedling those with radicle greater than 2.5 cm and presence of at least two sary roots. At 45 days after sowing counting of seedlings with opening of expanded cotyledons leaves was carried out and the results expressed in percentage.

The experiment was conducted in a completely randomized design, with five maturity stages (green, yellowish-green, cherry, over-ripe and dry) and four replications. The Assistat Version 7.5 Beta 2010 software was used for statistical data analysis, and the averages compared by Scott Knott test at 5% probability.

Gene expression

Initially, all materials used for RNA extraction were treated with diethyl pyrocarbonate solution (DEPC) at 0.5% to inactivate RNAses. The solutions were prepared with distilled milli-Q RNAse-free water. For RNA extraction was used Plant RNA Purification Reagent (Invitrogen) according to the manufacturer's manual. To each well containing approximately 100 mg of macerated tissue, 500 µL of cold reagent (4°C) was added and vortexed. Then the tubes were incubated for 5 min at room temperature, left in horizontal position for maximizing the RNA extraction. After this period, the material was subjected to centrifugation at room temperature, for 2 min at speed 12.000 g and supernatant was transferred to a new tube. It was added 100 µL of 5M NaCl and homogenized by vortexing. Thereafter, 300 µL of chloroform were added and the tubes were homogenized by inversion. For phase separation, the samples were subjected to centrifugation for 10 min at 4°C (12,000 g) and the upper aqueous phase was transferred to a new tube. Then an equivalent aqueous phase volume of cold isopropanol (approximately 400 µL) was added and stirred by vortexing for 5 s. The samples were kept at room temperature for 10 min and then also subjected to centrifugation for 10 min at 4°C (12,000 g). After having the supernatant was discarded, the pellet was washed with 1 mL cold 75% ethanol and the tubes were subjected to centrifugation for 1 min at room temperature (12,000 g). The residual liquid was removed from the tube with a pipette and the RNA was resuspended in 20 µL Milli-Q distilled RNAse-free water.

The quantity and quality of total RNA were evaluated on a Nanovue Plus (GE Healthcare Life Sciences) spectrophotometer. To evaluate the integrity of extracted samples, RNA was subjected to electrophoresis on 1.5% agarose gel stained with ethidium

bromide, subsequently visualized under ultraviolet light and the image captured by Kodak Photo documentation EDAS 290 System (Kodak®). The total RNA was treated with Ambion DNase I.

Subsequently, RNA was purified using RNeasy minelute cleanup (QIAGEN) kit and this purification step was performed to ensure the removal of genomic DNA and salts resulting from the extraction, which could compromise the PCR efficiency.

For cDNA synthesis the High Capacity cDNA Reverse Transcription (Applied Biosystems) kit was used. First, RNA was prepared to a concentration of 1µg in a final volume of 10 µL. After this step, a mix containing 2 µL of 10X enzyme buffer was prepared, 2 µL of RT Radom Primers 10X primer, 0.8 µL of dNTP (100 mM) mix, 1 µL of MultiScribe^{TM} Reverse Transcriptase, and water to a final volume of 10 µL/sample. To each prepared solution, 10 µL of 1 µg RNA, were added 10 µL of this mix. The tubes were subjected to the thermal cycler programmed with three steps: 10 min at 25°C for annealing of primers, 2 h at 37 fo action of the enzyme, and 5 min at 85 for inactivation. The samples were stored at -20°C in a freezer.

The cDNA was used as template for quantitative analysis of gene expression using the ABI PRISM 7500 Real-Time PCR (Applied Biosystems) by SYBR Green detection system.

Since the genes responsible for maturation of coffee beans are still little known, EST searches were performed with already identified Coffea sequences, as well as from other species deposited in the NCBI genbank database (Nacional Center for Biotechnology Information). Sequences were obtained from the database of sequences of Contigs, from the Coffee Genome Project, and the primers designed in the Primer Express 4.1 software, as shown in Table 1.

Table 1. Specific primers gibberellin ($CaGA_3$) and abscisic acid ($CaABI_3$) and endogenous controls ubiquitin (CaUBI) and 14-3-3 protein, used in real-time PCR reactions.

Gene	Forward (5' → 3')	Reverse (5' → 3')
CaGA3	TTGCTAACTCCCTTGCTGATCTT	CAACTGTTTCTCCTTCGCGAAT
CaABI3	GAAACGCATCTTCCTGAACTTGA	CCTATACCGCATGTTCCAGACA
CaUBI	CGCTGACTACAATATCCAAAAGGA	CTGCATTCCACCCCTCAGA
14-3-3 protein	TGTGCTCTTTAGCTTCCAAACG	CTTCACGAGACATATTGTCTTACTCAAA

First we used an absolute quantitation assay, where a standard curve was determined, together with the efficiency of primers and presented the best dilution for the samples. CDNAs were diluted in 1:5, 1:25, 1:125, 1:625, 1:325. After selection of 1: 5 dilution with primers efficiency between 94 to 97%, the relative expression by comparative CT method test was determined. All samples were performed with three replicates, including negative and endogenous controls. Expression of 14-3-3 gene, ubiquitin (CaUBI), $CaGA_3$ and $CaABI_3$ genes were analyzed.

The thermal conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, ending at 95°C with 1 s. Data were analyzed with v. 2.0.1 software, from the 7500 real-time PCR system (Applied Biosystems) at the Central Laboratory of Seed Analysis / Plant Science / UFLA. For each reaction it was used 2 µL of

cDNA, 10 mM of each primer, and 7.5 μL of SYBR® Green PCR Master Mix (Applied Biosystems) in a final volume of 15 μL / sample.

For calculation of expression, each sample was normalized with the endogenous control 14-3-3 and CaUBI, using the following equation:

$$\Delta Ct = Ct_{(TG)} - Ct_{(RGM)} \quad \text{(Eq. 1)}$$

$$\Delta\Delta Ct = \Delta Ct - \Delta Ct_{(control)} \quad \text{(Eq. 2)}$$

$$Ratio = 2^{-\Delta\Delta Ct} \quad \text{(Eq. 3)}$$

where, Ct is cycle threshold, TG is target gene, and RGM is the reference gene mean. Threshold was set automatically. The data are presented as means ± S.D. Significant differences ($P < 0.005$) between means were determined for each variable using the Scott Knot test to rank the experimental treatments.

RESULTS AND DISCUSSION

According to the data analysis, there were significant differences among the data for all variables (Table 2). Through results of tests determining the physiological quality, it can be seen that seeds harvested in cherry and over-ripe stages, had superior physiological potential than other stages of maturation, for all variables analyzed. Studies have shown that to obtain the best quality coffee seeds, it is recommended to harvest the fruit in the cherry stage (Veiga et al., 2007). At this stage, there is accumulation of storage proteins, sucrose and complex polysaccharides representing the main reserves of the seed (De Castro and Marraccini, 2006).

However, because it is a kind of intermediate behavior, the last stage of maturation, characterized by natural drying of seeds, can impair the physiological quality. Coffee seeds have been considered intermediate in terms of performance during storage (Ellis et al., 1990; Hong and Ellis, 1995; Eira et al., 1999), because they tolerate some dehydration compared to recalcitrant seeds and can present low-temperature sensitivity.

It was observed that green fruits seeds have low physiological quality, and germination and vigor have almost null values (Table 2). The yellowish-green seeds presented a germination potential, but the seed vigor was very low compared to the seeds at cherry and over-ripe stages. This is also observed in dry fruit seeds which have lost physiological quality during the natural process of drying (Table 2). These results are similar to those found by Veiga et al. (2007) and Rosa et al. (2011), in studies with coffee seeds, who found germination and vigor values of seeds harvested in the cherry stage higher than those of seeds harvested in the yellowish-green stage. Guimarães et al. (2002) also found that the vigor of coffee seeds increases markedly between the green and yellowish-green stages, as we also found.

Results of the physiological evaluation, root protrusion and strong normal seedlings carried out in the germination test, it was observed that the cherry and over-ripe seed stages aren't statistically different from each other. As for the results of

expanded cotyledonary leaves, there was a significant difference, with cherry seeds having greater vigor.

Table 2. Results of the physiological quality evaluation of coffee seeds harvested at different pre and post physiological maturity stages. Values in %.

Phenological stage	Water content	Root protrusion	Germination	Normal strong seedlings	Expanded cotyledonary leaves
Green	69.9	1 D	1 D	1 D	0 E
Yellowish-green	66.3	42 C	76 B	63 B	32 C
Cherry	68.5	89 A	82 A	88 A	58 A
Over-ripe	49.9	89 A	86 A	82 A	42 B
Dry	23.1	66 B	27 C	10 C	11 D

Means followed by the same letter in the column do not differ significantly at 5% probability by the Scott Knott test.

In gene expression analysis it was found for all the studied genes, expression pattern differences between parts of the seed (endosperm and embryo), intact seeds and maturation stages (Figure 1 and 2). These results illustrate the complexity of gene regulation in seed maturation. According to Gutierrez et al. (2007), the system of higher plants is divided into two phases: embryo and endosperm development, and seed maturation. For the selected genes in this study, it was expected that the expression was only found in the embryo. However, it was observed that these genes are also expressed in endosperm, as well as observed in earlier studies of expression of β-tubulin (TUB) and endo-β-mannanase (MAN) genes in coffee seeds (Santos et al., 2015). The use of whole seeds in the RNA extraction may have resulted in a high embryonic RNA dilution, since the embryo's volume is estimated to 1/40 of the whole seed (José et al., 2009).

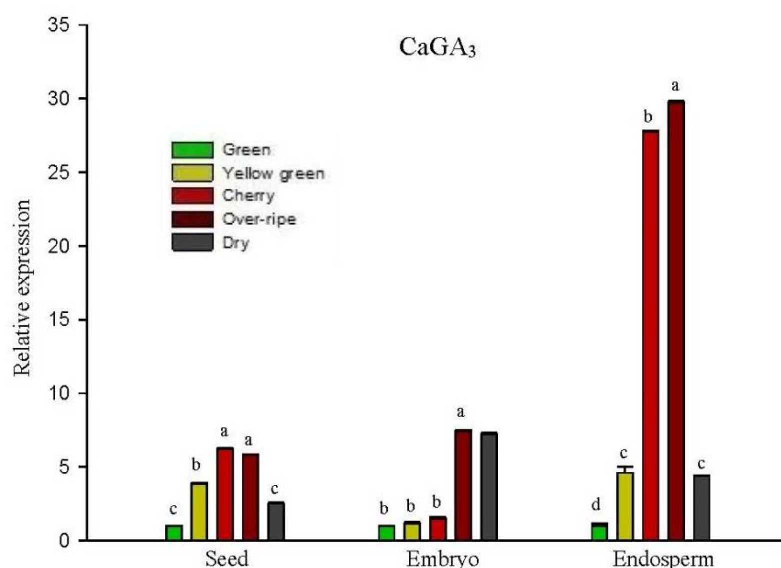

Figure 1. Quantification of $CaGA_3$ gene expression in coffee seeds at different pre and post physiological maturity stages and in different parts of the seed (intact seed, endosperm and embryo).

In Figure 1, there is greater expression of the $CaGA_3$ transcript in cherry and over-ripe stages in whole seeds and endosperm. However, in the embryo, expression was higher in raisin and dry stages. Overall, it was found that in the late stages of seed maturation process that there was increased expression in all parts of the seed (whole seed, embryo and endosperm). GA_3 plant hormone is important in numerous physiological processes such as germination, stem extension, leaf, floral and seed expansion development (Ogawa et al., 2003; Yamauchi et al., 2004; Holdsworthet al., 2008). Unlike ABA, the GA_3 is involved in breaking seed dormancy and is fundamental in seed germination in many species (Yamauchi et al., 2004).

The $CaGA_3$ expression results with peaks at cherry and raisin stages showed the same trend in the germination results (Table 2), where a higher average of normal seedlings for these same stages of maturation was observed.

Gibberellins determine significant physiological changes in plants, i.e., interfere flowering, sex expression, senescence, abscission, seed germination and breaking of dormancy. In coffee seeds specifically, it was observed that the presence of exogenous gibberellin accelerates the germination inducing action of enzymes which promote softening of capillary endosperm (Castro et al., 2005, Silva et al., 2005), allowing root protrusion.

Figure 2 illustrates the results of $CaABI_3$ gene expression on the biosynthesis of abscisic acid in different parts of coffee seeds, derived from fruits harvested during the described maturation stages. Increased expression of $CaABI_3$ gene was observed in the late stage of maturation, cherry and over-ripe in endosperm (Figure 2).

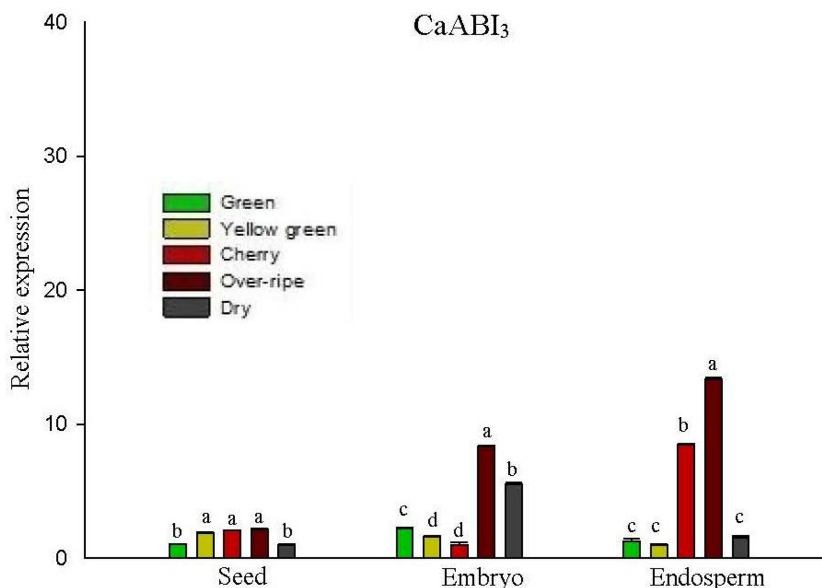

Figure 2: Quantification of $CaABI_3$ gene expression in coffee seeds at different pre and post physiological maturity stages and in different parts of the seed (intact seed, endosperm and embryo).

In intact seeds, the $CaABI_3$ gene expression was relatively low when compared to the expression observed in embryos and endosperms. In embryos, it was observed higher gene expression in raisin stage followed by dry stage. Molecular studies on seed maturation in model species revealed that maturation is primarily driven by three key genes: ABA INSENSITIVE 3 (ABI_3), FUSCA 3 (FUS3), LEAFY COLTILEDON 1 (LEC1) and LEAFY COLTILEDON 2 (LEC2) (To et al., 2006; José et al., 2009). In peas, it was observed that $PsABI_3$ is expressed in late stages, together with storage protein synthesis and delayed maturation (Radchuk et al. 2010), showing the involvement of abscisic acid in the final maturation process.

In coffee, abscisic acid regulates the germination of seeds. Through the application of exogenous ABA it was observed reduced abundance of microtubules, inhibition of embryo cells growth, reorganization of microtubules and DNA replication in embryonic axis, delaying the germination (Silva et al., 2008).

In the present study, it was observed a negative relationship between $CaABI_3$ expression in the embryo and seed physiological quality, especially in seeds coming from harvested fruits in cherry stages, which had high germination values and low levels of $CaABI_3$ expression. This result was also observed in seeds of different species like Arabidopsis (Reyes & Chua, 2007), lettuce (Clemente et al., 2013), beet (Hermann et al., 2007) among others.

In addition to regulating the germination process, ABA plays an important role in numerous physiological processes such as seed maturation, regulation of growth and development, seed dormancy and is also responsible for adaptation to environments with stress condition (Liu et al., 2010). ABA levels are low during embryogenesis and increases significantly with the progress of maturity. Thus, as the embryo enters the maturation phase, the ABA content increases, and the result of ABA / GA high ratio promotes maturation, induces dormancy and inhibit embryo growth and germination (Santos-Mendonza et al., 2008; Radchuk et al., 2010).

CONCLUSIONS

The highest expression of $CaGA_3$ gene occurs in the cherry and over-ripe stages of coffee seeds, corresponding to a better physiological performance.

ABI_3 gene expression linked to the biosynthesis of abscisic acid is higher in the over-ripe and dry stages of coffee seeds.

There are differences in the $CaABI_3$ and $CaGA_3$ gene expression in embryos and endosperm of coffee seeds.

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