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PLANT TISSUE CULTURE

A putative *BABY BOOM-like* gene (*CaBBM*) is expressed in embryogenic calli and embryogenic cell suspension culture of *Coffea arabica* L

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Abstract The acquisition of embryogenic cell suspension (ECS) cultures has been one of the main objectives to maximize clonal propagation of the coffee plant. However, the majority of somatic embryogenesis induction requirements are genotype-dependent. Therefore, molecular markers linked to the embryogenic transition events may be useful. The *BABY BOOM* (*BBM*) gene can be considered as one of those markers, as it is related to the embryogenic process and to cell proliferation. *BBM* homologous sequences were obtained from Expressed Sequence Tags (ESTs) in a databank generated by the Brazilian Coffee Genome Project. We selected EST-contigs that showed similarities with *BBM* sequence from different species. Two EST-contigs (C2 and C9) were expressed *in silico* in cellular suspension libraries and embryogenic calli of coffee. Contig C9, defined as *BBM*-like (*CaBBM*), presented similarity with *BBM* genes and showed 2-fold change in expression in ECS relative to embryogenic calli (EC). Contig C2, on the other hand, was related to the ERF-like family. It showed basal expression in non-embryogenic calli (NEC) and approximately 66- and 311-fold less in ECS and EC compared with *CaBBM* in the same samples, respectively. These data suggest that *CaBBM* is likely to be a *BBM* ortholog in *Coffea arabica*, which has potential for use as a molecular marker to further increase the methodological efficiency of *in vitro* culture of coffee.

Keywords Baby Boom · Embryogenic cell suspensions · EST-contigs · Gene expression

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Introduction

Somatic embryogenesis, the formation of the embryo originating from somatic cells, is a natural asexual reproduction mechanism in some plant species that can be induced *in vitro*. However, the experimental conditions that induce somatic cells to acquire embryogenic competence are specific to the genotype, tissue type, and development phase of the plant from which the explant is obtained, which makes protocol acquisition practically empirical (Jiménez 2001; Fehér *et al.* 2003; Namasivayam 2007).

Cells with embryogenic potential are characterized as being small, isodiametric, having a big nucleus, evident nucleoli, dense cytoplasm, microvacuoles, and being rich in amyloplasts (Domergue *et al.* 2000; Quiroz-Figueroa *et al.* 2006; Namasivayam 2007), thus resembling the meristematic cells (Guerra *et al.* 1999). In fact, embryogenic cell groupings contained in banana cell suspensions, the cells that form the embryo (embryonic), have few amyloplasts. Rather they are surrounded by typical embryogenic cells (Domergue *et al.* 2000), establishing a source-sink relationship among the cells inside the meristematic region. This data was used to classify and evaluate the quality of embryonic cell suspensions (ECSs) in banana, but it did not allow detection of the molecular events that induce the morphological differentiation associated with embryogenic potential, which could be an even more fundamental factor of efficiency.

In general, the *in vitro* embryogenic process is initiated by auxins or cytokinins that promote the proliferation of undifferentiated cells. Subsequently, auxins should be removed to allow induction of embryogenesis and consequent protoderm development. Associated periclinal cell divisions result in tissue invagination and establishment of the embryonic axis (Toonen *et al.* 1994; Arnold 2008). In the process, different groups of genes are expressed in a coordinated way to conduct the vegetative changes required for embryogenic

development. Overexpression of these genes is correlated with an increase of the embryogenic capacity by the somatic cells. These genes possibly are regulated by the *BBM* gene (Boutilier *et al.* 2002; Passarinho *et al.* 2008).

BBM encodes a plant-specific transcription factor belonging to the AP2/ERF superfamily that is related to embryogenic processes and cellular proliferation in meristematic regions. When overexpressed in *Arabidopsis thaliana*, *Brassica napus* (Boutilier *et al.* 2002), or *Glycine max* (El Ouakfaoui *et al.* 2010), *BBM* induces spontaneous formation of embryogenic calli and somatic embryos without addition of plant growth regulators. *AINTEGUMENTA* (*ANT*), also a member of AP2/ERF family that regulates growth and cell number during organogenesis (Mizukami and Fischer 2000), produces the same effect as *BBM* when overexpressed in *Arabidopsis*, and when suppressed, it provokes a reduction in the calli cell count (Elliott *et al.* 1996; Klucher *et al.* 1996).

The AP2/ERF domain family of transcription factors is found extensively throughout the plant kingdom, being present in mosses, algae, gymnosperms, and angiosperms. It also represents the second largest group of transcription factors in plants, with up to 200 members in a single genome (Nole-Wilson *et al.* 2005; Shigyo *et al.* 2006). The AP2/ERF domain is a DNA binding domain of approximately 60 to 70 amino acids (Okamuro *et al.* 1997) that was initially identified in the *Arabidopsis APETALA2* (*AP2*) and *ETHYLENE RESPONSE FACTOR* genes (Jofuku *et al.* 1994; Ohme-Takagi and Shinshi 1995; Weigel 1995; Okamuro *et al.* 1997). The superfamily includes all genes encoding at least one AP2/ERF domain (Licausi *et al.* 2010). The AP2 subfamily of proteins contains two AP2/ERF domains separated by a linker region (Zhou *et al.* 1997; Riechmann and Meyerowitz 1998). *BBM* proteins belong to the AP2 subfamily, whose members include *AP2* (Jofuku *et al.* 1994), *indeterminate spikelet1* (*ids1*; (Chuck *et al.* 1998), *ANT* (Elliott *et al.* 1996; Klucher *et al.* 1996), *Glossy15* (*Gl15*; Moose and Sisco 1996), and the maize gene *ZmMHCF1* (Daniell *et al.* 1996). AP2/ERF-domain proteins regulate two major and vital processes in plants: response to stress and control of growth and development. While members of the AP2 subfamily regulate embryogenic and developmental processes, the members of the ERF subfamily are related to biotic and abiotic stress responses (Stockinger *et al.* 1997; Zhou *et al.* 1997; Liu *et al.* 1998; Menke *et al.* 1999).

Recent work has shown that the use of molecular markers has facilitated a better understanding of the genetic basis of important traits in coffee (Leroy *et al.* 2005). In this context, possible *BBM* homologs were identified *in silico* and quantitatively expressed (quantitative reverse transcription (qRT-PCR) in materials histologically qualified as embryogenic calli (EC), non-embryogenic calli (NEC), and embryogenic cell suspensions (ECSs) of *Coffea arabica* cv. 'Catiguá' with the objective of evaluating the use of *BBM*-related genes as potential molecular markers for the acquisition of embryogenic competence with a view to improve current protocols for EC and ECS.

Material and Methods

Search for sequences homologous to BBM. BBM gene sequences were obtained from the Expressed Sequence Tag (EST) bank generated by the Brazilian Coffee Genome Project (Vieira *et al.* 2006). Through the Gene Project interface (http://www.lge.ibi.unicamp.br/cafe), it was possible to search for sequence reads and to form clusters from the associated reads, using a combination of keywords "*BBM*," "*Baby Boom*," and the Basic Local Alignment Search Tool (Altschul *et al.* 1997) of nucleotide (BLASTn) and amino acid (tBLASTn) sequences of four *BBM* homologous genes (accessions: EF687843.1, AY899909.1, AF317907, and AK065701.1) deposited in the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov). From this strategy, we found 113 reads that showed significant alignment (e-value $>10^{-4}$). Those reads were grouped into clusters, forming 19 EST-contigs. Amino acid sequences of those EST-contigs were deduced using the ExPASY interface (http://ca.expasy.org/tools/dna.html), and the integrity of the AP2/ERF domains was verified using the NCBI Conserved Domain Search program. Sequences with the conserved AP2/ERF domains were compared with the *BBM* sequences using the BLASTx algorithm. Nine EST-contigs that presented similarity were grouped in a dendrogram with *BBM* sequences (AP2 subfamily) and members of the ERF subfamily and analyzed as to the gene expression *in silico* in the Brazilian Coffee Genome Project libraries.

Similarity dendrogram. The sequences of nine selected ESTs-contigs were aligned (ClustalW; Thompson *et al.* 1994) and grouped (MEGA 4; Tamura *et al.* 2007) using the neighbor-joining comparison model (Saitou and Nei 1987) by the *p* distance and pairwise suppression method. The validity of the dendrogram as to the distance of the clusters was given by the probabilistic bootstrap test (Sitnikova *et al.* 1995).

In silico gene expression—electronic northern. For analysis of *in silico* gene expression, the frequencies of the reads that formed each expressed EST-contig in the libraries were normalized, since the libraries were not the same size. The normalization consisted of multiplying the frequency of each read by the ratio between the total number of reads from all the libraries and the number of reads of each library in which the given read was expressed. With the normalization results, a matrix was processed using the Cluster and TreeView programs (Eisen *et al.* 1998), in which the libraries and related EST-contigs were grouped by hierarchical clustering. From

the *in silico* analysis (similarity dendrogram and electronic northern), two EST-contigs (C2 and C9), expressed *in silico* specifically in embryogenic tissues, were then analyzed quantitatively in plant materials.

Identification of common grouping motifs. The Multiple Expectation Minimization for Motif Elicitation program (MEMME; http://meme.nbcr.net/meme/) version 4.9.0 (Bailey *et al.* 2006) was used to elucidate grouping motifs among *BBM* genes (Boutilier *et al.* 2002; Imin *et al.* 2007; El Ouakfaoui *et al.* 2010) and the sequences of the selected EST-contig (C9). Any number of replications, maximum number of motifs set to 7, and optimum width between 50 and 300 were used as parameters. Motif annotation regarding the functional domains present in *BBM* sequences was carried out using the NCBI Conserved Domain Search program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Plant material. The plant materials used for expression analysis included embryogenic calli (EC), non-embryogenic calli (NEC), and embryogenic cell suspensions (ECSs). These materials were harvested after 5 mo of *in vitro* cultivation of foliar explants of *C. arabica* cv. 'Catiguá,' according to the previously established protocols (Berthouly and Michaux-Ferriere 1996; Teixeira *et al.* 2004), and thus were characterized from histological analyses. The research was conducted at the Laboratório Central de Biologia Molecular (LCBM) of Universidade Federal de Lavras (UFLA), Brazil.

Histological characterization. Samples of the plant materials were fixed with FAA_{50} (4% formaldehyde + 5% acetic acid + 50% ethanol, *v*/*v*) for 48 h at room temperature, dehydrated in ethanol series (60 to 100%), and embedded in epoxy resin (Historesin®, Leica, Nußloch, Germany) according to manufacturer's protocol. Five-micron sections were obtained using a manual rotary microtome (Reichert-Jung, 1130), stained with 0.05% (*w*/*v*) toluidine blue, and observed under a light microscope (Zeiss®, Axio Scope, Jena, Germany), and the images were captured by Axiovision Rel. 4.8 software with standard parameters.

Quantification of gene expression. The expression of the candidate genes (EST-contigs C2 and C9) were quantified by qRT-PCR with primers (Table 1) designed using the Primer Express 3.0 program (Applied Biosystems, Foster City, CA) from EST-contig sequences, selected by *in silico* analysis. As an endogenous control, primers for the *ACTIN* and *GAPDH* coffee genes (Table 1) were used (Barsalobres-Cavallari *et al.* 2009). For the assays, total RNA of EC, NEC, and ECS was extracted from 25 mg of tissue with three biological repetitions using the NucleoSpin® Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol, and RNA integrity was checked by electrophoresis in agarose gels and ethidium bromide staining. The absence of DNA was verified by PCR with the *ACTIN* endogenous control primer (Barsalobres-Cavallari *et al.* 2009), and the RNA samples were also quantified by spectrophotometry (NanoDrop 1000, Wilmington, DE), determined by the ratio between A260/A280 and A260/A230. The RNA biological replicates were grouped in an RNA pool at a final concentration of 100 ng/μL. Next, complementary DNAs (cDNAs) were synthesized in reverse transcription reactions using the High-Capacity® Kit (Applied Biosystems) according to manufacturer's protocol. Those reactions were conducted with 10 μL of the kit reaction mix + 10 μL of the RNA pool (1 μg) for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. With the obtained cDNAs, amplifications were carried out (ABI PRISM 7500 Real-Time PCR; software version 2.0.1 [Applied Biosystems]) using SYBR® Green, according to manufacturer's protocol. Each amplifications used ~10 ng of cDNA and 1 μg of primer for 5 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 concluded for 15 s at 95°C. The expression data, resulting from the amplifications, were normalized ($\Delta CT = CT_{\text{target gene}} - CT_{\text{endogenous control}}$) and relatively quantified ($RQ = 2^{-\Delta\Delta CT}$) based on CT (cycle threshold) of the expression of the endogenous controls *ACTIN* and *GAPDH* (calibrator) ($\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{calibrator}}$) present in the same reaction. The efficiency of the reactions was verified by the equation $(1+E) = 10^{(-1/\text{slope})}$ (Ramakers *et al.* 2003). All the primers pairs had efficiency higher than 0.90.

Results and Discussion

Previous studies have aimed to optimize the protocol for inducing somatic embryogenesis in *Coffea* ssp. using molecular markers. Although differences in expression at both RNA and protein levels were observed, no variation at the DNA level was detected by amplified fragment-length polymorphisms (AFLP) that could explain the disparity in embryogenic competence (Sanchez-Teyer *et al.* 2003). *BBM* has demonstrated involvement in somatic embryogenesis and cellular proliferation in meristematic regions (Boutilier *et al.* 2002; El Ouakfaoui *et al.* 2010). We therefore proposed that differential gene expression may modulate the embryogenic capacity of *in vitro*-cultured coffee cells and identified a *BBM* homolog from *C. arabica* that could be used a putative molecular marker by qRT-PCR to assist in the optimization of the regeneration protocol.

Analysis of possible homologous sequences. *In silico* searches resulted in obtaining 19 EST contigs with significant similarity (e-value $>10^{-4}$) to *BBM*. Of these, nine presented complete AP2/ERF domains and were expressed in coffee plant

Table 1 Candidate *BBM*-like and reference primer sequences used for quantitative PCR analysis

Gene name	Source gene	Gene description	Primer sequence[z] (5′-3′)	Amplicon length (bp)
Actin[x]	SGN-U353034	Actin 7	AATTGTCCGTGACATCAAGGAA TGAGCTGCTCTTGGCTGTTTC	82
GAPDH[x]	SGN-U347734	Glyceraldehyde-3-phosphate dehydrogenase	TTGAAGGGCGGTGCAAA AACATGGGTGCATCCTTGCT	59
CaERF-like	Contig 2[y]	One AP2 domain (ERF-like transcription factor)	CGAAACTTGTCACGATCCACAT ACCCTTGGGAAGGACTTGCT	61
CaBBM	Contig 9[y]	*BBM*-like; two AP2 domain (AP2-like transcription factor)	TACGAAGGAAGAGTAGTGGGTTTTC TGGTGTCTTGTCACTCCTCGAT	64

[x] Primer sequence (Barsalobres-Cavallari *et al.* 2009)

[y] Gene accession according expression *in silico* in coffee plant embryogenic calli and cellular suspension libraries

[z] Forward (*upper line*) and reverse (*lower line*) primer sequences

embryogenic libraries (Vieira *et al.* 2006), and two (C2 and C9) showed exclusive expression in the EC and cellular suspension libraries (Fig. 1).

AP2/ERF domain proteins have a role in two important processes in plants: response to stress and control of growth and development. The contig C2 sequence, 824 bp in length, was derived from alignment of six ESTs: four from the EA1, IA1, and IA2 libraries (EC) and two from the CB1 library (suspension cells treated with acibenzolar-S-methyl and brassinosteroids). The *in silico* expression pattern for C2 is shown in Fig. 1. It presented with a single AP2 domain, grouping with the ERF subfamily of proteins (Fig. 2). On the other hand, contig C9, 1,726 bp in length, was formed from four ESTs: three from the EA1, IA1, and IA2 libraries (EC) and one from the RT8 library (cells in suspension in the presence of aluminum) and showed significant homology to *BBM* genes from *Arabidopsis* (Boutilier *et al.* 2002), *G. max* (El Ouakfaoui *et al.* 2010), and *M. truncatula* (Imin *et al.* 2007; Fig. 3). As shown in Fig. 2, it also grouped with other members of the AP2 subfamily, i.e., *ANT* (Elliott *et al.* 1996; Klucher *et al.* 1996) and *AP2* of *Populus trichocarpa* (XP_002316179.1). Consistently, C9 is predicted to contain two AP2/ERF domains (Fig. 3), typical of the AP2 subfamily of proteins (Riechmann and Meyerowitz 1998). Remarkably, ANT-like proteins have been shown to function exclusively in pathways related to development (Nole-Wilson *et al.* 2005; Mudunkothge and Krizek 2012). Taken together, these results

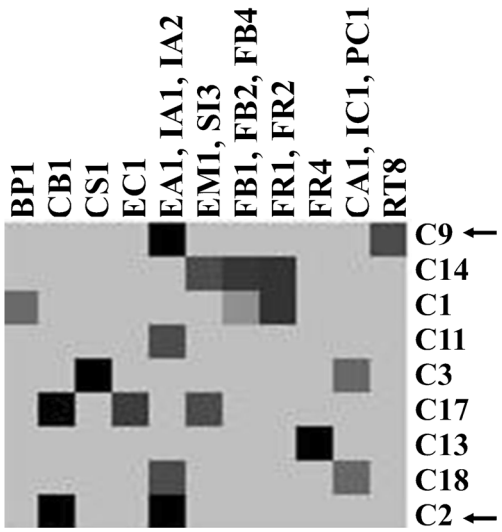

Figure 1. Electronic northern representing EST-contigs expression levels in the coffee libraries (the darker the gray tones, the higher the expression). *BP1*, cells in suspension treated with acibenzolar-S-methyl; *CB1*, cells in suspension treated with acibenzolar-S-methyl and brassinosteroids; *CS1*, cells in suspension treated with NaCl; *EC1*, embryogenic calli of *C. canephora*; *EA1*, *IA1*, and *IA2* embryogenic callus; *EM1* and *SI3*, germinating seeds (whole seeds and zygotic embryos); *FB1*, *FB2*, and *FB4*, floral buds in different development stages; *FR1* and *FR2*, floral buds + fruitlets at the first stage + fruits at different stages; *FR4*, fruit (*C. racemosa*); *CA1*, *IC1*, and *PC1*, non-embryogenic calli with and without 2,4-D; *RT8*, root and cells in suspension in the presence of aluminum.

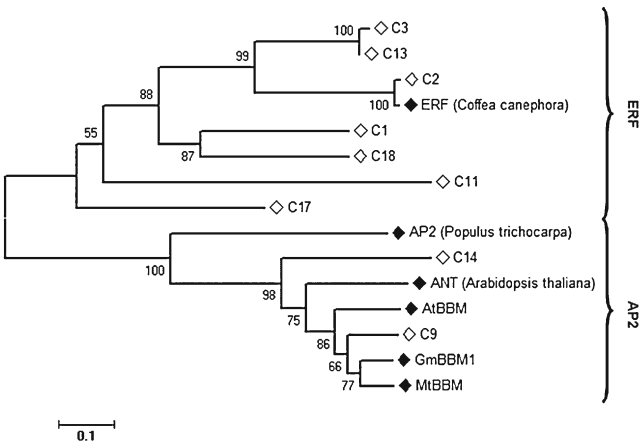

Figure 2. Similarity dendrogram between amino acid candidate sequences for *BBM* and the ERF and AP2 subfamilies. EST-contig sequences (*open diamond*); NCBI sequences (*closed diamond*); bootstrap values less than 50% were omitted.

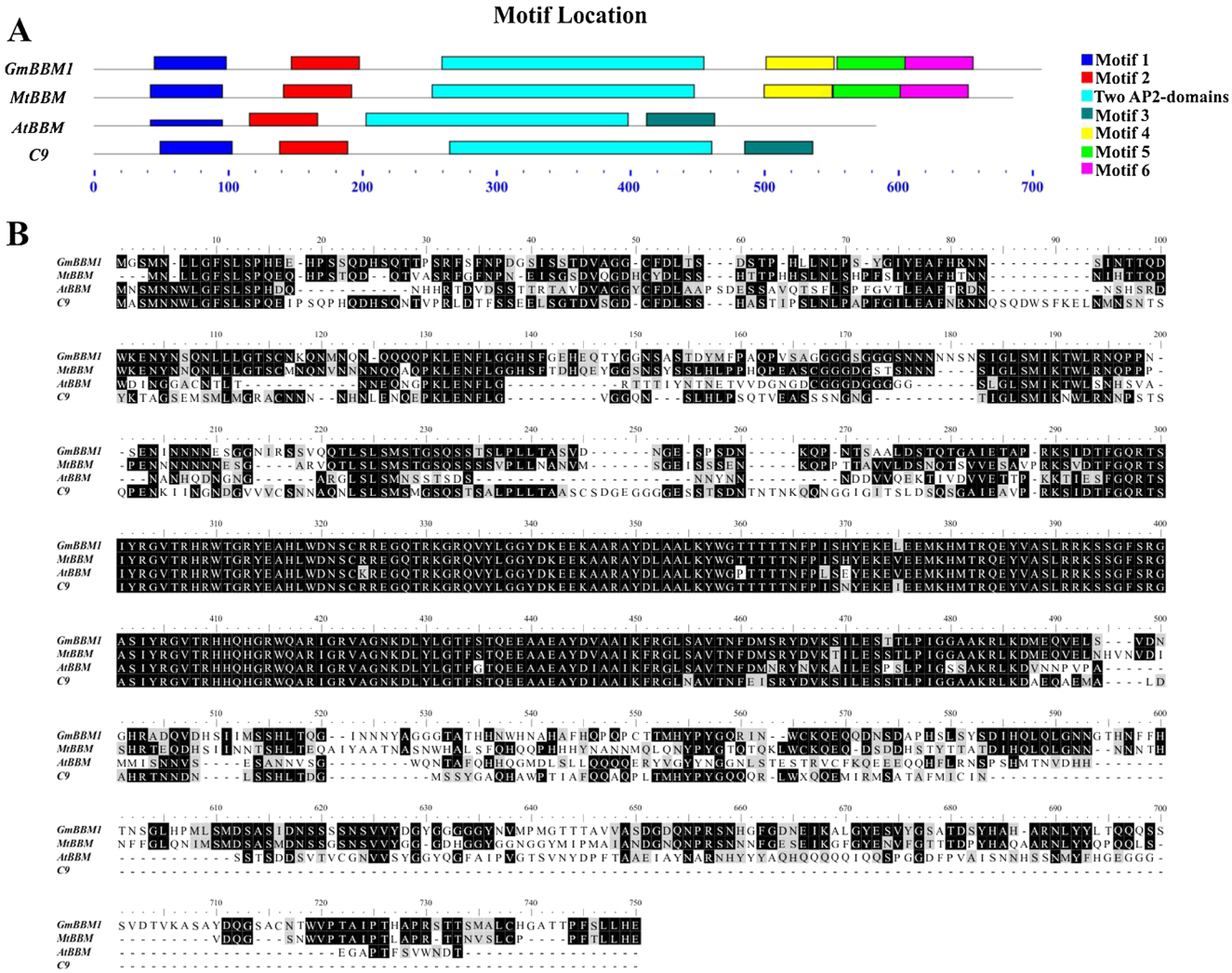

Figure 3. *A* Putative domains of the C9 protein (CaBBM) compared to other BBM proteins. The different domains are indicated by *different colors. B* Multiple alignment of C9 amino acid deduced sequences with homologs GmBBM1, MtBBM, and AtBBM showing homology in the two AP2/ERF domains (AP2 subfamily). The residues marked in *black* indicate 100% identity.

suggest that C9 (termed *CaBBM*) is a potential candidate as a molecular marker, being related to somatic embryogenesis in *C. arabica*.

Histological characterization. In *C. arabica*, development of indirect somatic embryogenesis is sporadic within a callus clump, yet it is possible to identify different histologically and morphologically discrete stages (Fig. 4). Clusters of somatic embryos at different stages of development were used to establish ECS (Fig. 4*C*), including globular, heart-shaped, and torpedo phases (Fig. 4*D*). These calli had a creamy yellow coloration and a granular and brittle structure (Fig. 4*A*) and are considered suitable for the establishment of cellular suspensions because they have been shown to initiate somatic embryos at a high frequency in coffee (Quiroz-Figueroa *et al.* 2002; Teixeira *et al.* 2004) and banana (Domergue *et al.* 2000; Strosse *et al.* 2003). NEC, on the other hand, are of crystalline and aqueous structure (Fig. 4*B*) and present a large amount of vacuolated cells, symptomatic of the degeneration process (Fig. 4*F*). A cluster of embryogenic cells, densely stained with toluidine blue (Fig. 4*E*, *G*), may represent the first stage in the formation of somatic embryos, the pro-embryoid stage (Bieysse *et al.* 1993). Embryogenic cells are starch-rich, are highly mitotic, and are restricted to the callus surface. The differentiation extends to the entire callus and is characterized by the formation of pro-embryos that are moderately rich in storage compounds (Berthouly and Michaux-Ferriere 1996).

Gene expression analysis by qRT-PCR. Based on the *in silico* analysis, primers were designed for EST-contigs sequences C2 (*CaERF-like*) and C9 (*CaBBM*) to quantify their *in vitro* expression in ECS, EC, and NEC. The results showed the following: (i) variable *ERF-like* expression in all plant tissues tested, with higher expression in ECS (Fig. 5) and (ii) high

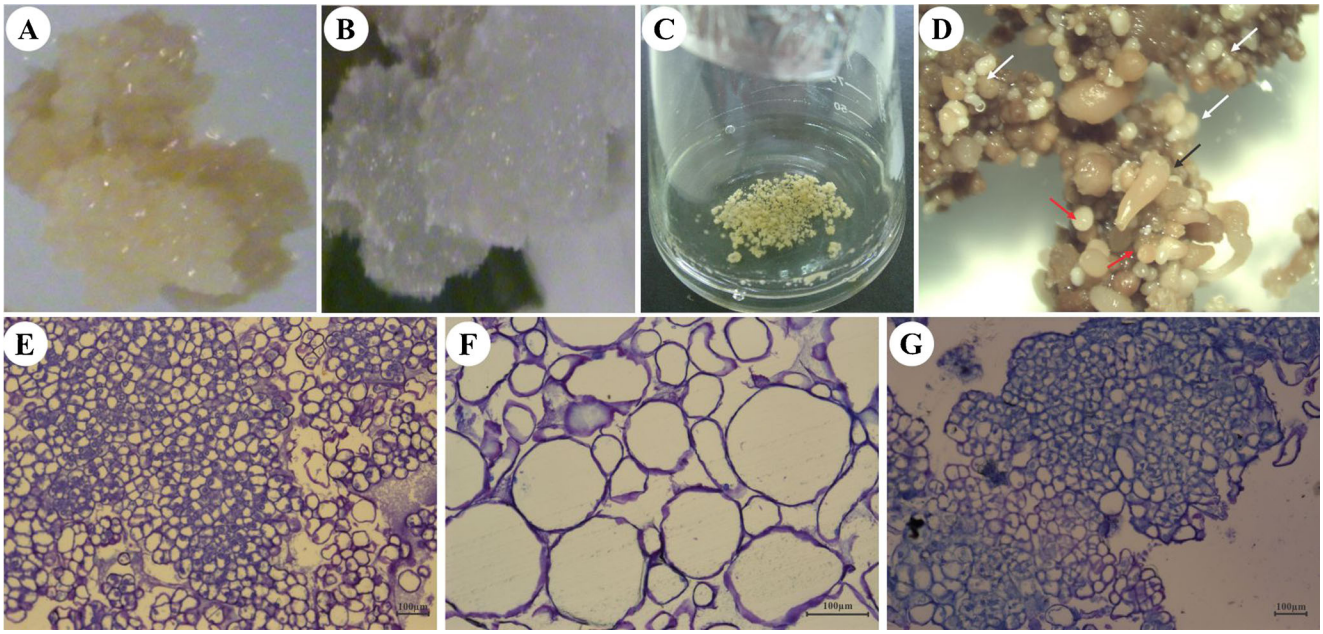

Figure 4. Developmental stages of somatic embryos obtained by indirect embryogenesis. *A* Morphology of embryogenic callus (EC) and *B* non-embryogenic callus (NEC) after 5-mo cultivation. *C* Morphology of embryogenic cell suspensions (ECS). *D* Embryos emerging from somatic ECS. A cluster of globular embryos (*white arrows*), heart-shaped embryos (*red arrows*), and torpedo stage embryo (*black arrow*). *E–G* Histological sections. *E* A prominent nucleus and dense cytoplasm of an EC, *F* vacuolated cells of NEC, and *G* heterogeneous nature of ECS.

CaBBM expression exclusively in embryogenic tissues, being 2-fold higher in EC than in ECS. Furthermore, C9 was more highly expressed, being 66- and 311-fold higher than the *CaERF-like* gene (C2) in ECS and EC, respectively (Fig. 6).

The *CaERF-like* gene represents an AP2/ERF superfamily member with 100% similarity to an *AP2/ERF* sequence from *Coffea canephora* fruit (Bustamante-Porras *et al.* 2005). Proteins with one AP2/ERF domain (ERF domain) are usually involved in biotic and abiotic stress processes (Wang *et al.* 2002). However, the *C. canephora* ERF gene isolated by Bustamante-Porras *et al.* (2005) grouped with genes expressed in cellular suspension of *Solanum tuberosum* (AAC29516), *Fagus sylvatica* seed (CAD21849), *Lycopersicon esculentum* (AAO34703), and *Prunus armenica* fruit (AAC24587). According to Bustamante-Porras *et al.* (2005), *C. canephora* ERF is related to the cellular

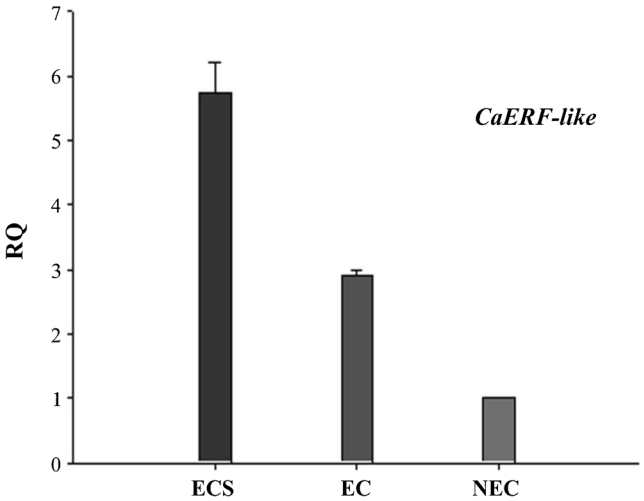

Figure 5. Relative quantitative expression (*RQ*) of *CaERF-like* in embryogenic cell suspensions (*ECS*), embryogenic calli (*EC*), and non-embryogenic calli (*NEC*) of *C. arabica* cv. 'Catiguá.' Expression values=average of technical responses; endogenous controls=*ACTIN* and *GAPDH*; *NEC*, reference sample.

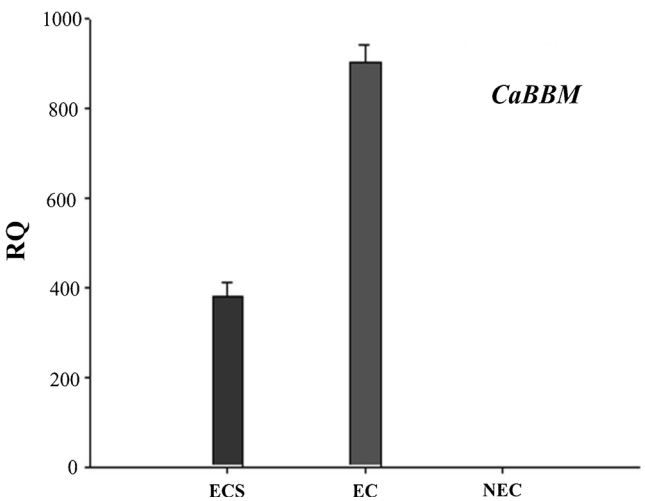

Figure 6. Relative quantitative expression (*RQ*) *CaBBM* in embryogenic cell suspensions (*ECS*), embryogenic calli (*EC*), and non-embryogenic calli (*NEC*) of *C. arabica* cv. 'Catiguá.' Expression values=average of technical responses; endogenous controls=*ACTIN* and *GAPDH*; reference sample=NEC.

differentiation or ripening processes, but not to plant defense responses. Even so, the role of this gene in fruit ripening and/or cell growth awaits confirmation.

In this study, the *CaERF-like* gene, C2, was expressed *in silico* in EC libraries (four ESTs) and cellular suspension libraries of *C. arabica* treated with acibenzolar-S-methyl and brassinosteroids (2 ESTs), but not in the fruit libraries tested (Vieira *et al.* 2006; Fig. 1). The cellular signaling pathway for brassinosteroids is related to defense against pathogens (Albrecht *et al.* 2008). However, there is no clear relationship between the pathway and the *in vitro* expression of *CaERF-like* in the embryogenic tissues of *C. arabica* cv. 'Catiguá' at the different developmental stages (Fig. 5). Therefore, it is possible that the gene function of *CaERF-like* is related to the cellular differentiation process in general and not solely associated with ripening. Consistent the hypothesis proposed by Bustamante-Porras *et al.* (2005) that this gene has a role in the cell growth, it may be expressed once a high level of differentiation related to somatic embryogenesis has occurred. Although this gene has shown expression in cellular suspension cultures *in silico*, which may indicate a role in cellular growth, it must be noted that the samples that showed expression had been exposed to treatments with various plant growth regulators. Therefore, expression may have been associated with stress responses, which are known to induce expression of ERF members (Shi *et al.* 2014). *MtSERF1* is a gene member of AP2/ERF superfamily in *M. truncatula*, and also, it is highly expressed in embryonic tissue and poorly expressed in NEC (Mantiri *et al.* 2008). Taken together, we propose that *CaERF-like* has a role in two different pathways, cell growth, and response to stress. The role of this gene in these pathways is now subjected to further studies for confirmation.

CaBBM seems to be related more directly with the embryogenic process, because this gene has the following: (i) two AP2 subfamily/domains (Fig. 3); (ii) similarity with the members of this subfamily that has been confirmed with a role in embryogenesis including *AtBBM* (Boutilier *et al.* 2002), *GmBBM* (El Ouakfaoui *et al.* 2010), and *MtBBM* (Imin *et al.* 2007; Fig. 3*B*); (iii) *in silico* expression in EC (EA1, IA1, and IA2 libraries) and cellular suspension libraries (RT8; Fig. 1); and (iv) expression in *in vitro* ECS and EC, but not NEC of *C. arabica cv.* 'Catiguá' (Fig. 6). Similar to *CaBBM*, *BBM* transcripts in *B. napus* and *A. thaliana* were detected in embryogenic cultures and subsequently in somatic embryos, but not in non-embryogenic cultures (Boutilier *et al.* 2002). However, considering that many genes are active in embryogenic tissues (Passarinho *et al.* 2008), the high level of *CaBBM* expression in EC was not unexpected, but it was surprising that expression was not equivalent in ECS, both *in silico* (Fig. 1) and *in vitro* (Fig. 6), since the cellular proliferation rate in ECS is higher than in calli and *BBM* is known to be associated with cellular proliferation (Passarinho *et al.* 2008; El Ouakfaoui *et al.* 2010). A possible explanation of the qRT-PCR expression levels of *CaBBM* in EC and ECS (Fig. 6) may be associated with the heterogeneous nature of the ECS samples (Fig. 4), where only a portion of the callus is embryogenic while the majority of cells are quiescent or undergoing degradation.

These data suggest that EST-contig C9 is the ortholog of *BBM* in *C. arabica* and has been termed *CaBBM* accordingly. Therefore, we propose that *CaBBM* or its promoter may be utilized as a molecular marker in coffee during the *in vitro* embryogenic process and may be a good parameter to evaluate the acquisition of embryogenic capacity of tissues undergoing differentiation. In this way, it may help to improve the methodological efficiency for obtaining ECS in coffee. Over time, it may be able to predict the optimal time to start ECS lines from the EC, such as when *CaBBM* is highly expressed, to significantly improve the embryogenic competence of this type of cell culture.

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