#### PLANT TISSUE CULTURE



# Molecular analysis of ERF subfamily genes during coffee somatic embryogenesis

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#### Abstract

With ample cultivation and economic potential, coffee is one of the main commodities in the world. Despite its vast cultivation, there remain problems related to its productivity as a consequence of pests and diseases, questions regarding its physiology, and difficulty with its propagation. Due to such challenges, the application of biotechnological tools, such as tissue culture and molecular markers, has become essential in the search for improvements to coffee cultivation. Among tissue culture techniques, somatic embryogenesis is considered the best form of micropropagation *in vitro* since it is possible to produce plants that are identical to the parent plant. It is therefore essential to identify the genes involved in the process of somatic embryogenesis, as well as to understand their functions. Different studies have shown the expression of *ethylene response factor* (*ERF*) genes in different tissues and under different conditions, mainly in response to biotic and abiotic stresses. However, little is known about their role during somatic embryogenesis. Thus, this study is aimed to identify and analyze the expression patterns of ERF subfamily genes during *Coffea arabica* L. somatic embryogenesis. The EST-*contig6*, 9, and 27 were confirmed to be the ethylene response factor 8, 13, and 12 genes from *Coffea arabica* L., respectively, and their relative expression analysis suggests that they may be involved in coffee somatic embryogenesis. The expression patterns of ethylene response factor 8 and 13 in embryogenic calluses and embryogenic cell suspensions showed that these genes can potentially act as somatic embryogenic markers.

Keywords Coffea arabica L. · Embryogenic potential · RT-qPCR · Transcriptional analysis · Molecular marker

## Introduction

Coffee has become one of the main commodities in the world due to its economic potential and broad cultivation (MAPA: Ministério da Agricultura, Pecuária e Abastecimento, 2017). Despite extensive cultivation and research on the species, due to its economic relevance,

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there are still problems related to its productivity as a consequence of pests and diseases (Carvalho *et al.* 2012; Carvalho *et al.* 2017), questions regarding its physiology and genetics (Barreto *et al.* 2012; Ságio *et al.* 2014), and difficulty with its propagation (da Rosa *et al.* 2011; Huang *et al.* 2014). In order to solve or minimize the problems related to coffee cultivation, breeding programs have sought the development of plants with superior characteristics; however, the time for the emergence of new and improved cultivars can be long, making it an obstacle to the process (Carvalho *et al.* 1991; Tonietto *et al.* 2012).

In this way, the application of biotechnological techniques, such as tissue culture and the use of molecular markers, can serve as powerful tools to aid the genetic improvement of coffee. Through *in vitro* micropropagation, it is possible to obtain thousands of seedlings with the characteristics of interest by means of a single leaf explant, an achievement that is almost impossible with propagation *via* seed (Carvalho *et al.* 1991; Campos *et al.* 2017). The use of molecular markers allows the identification of genetic

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patterns, which are important in the selection of genetic material with desirable characteristics. Such markers do not represent the target genes themselves, but act as flags that indicate their existence (Collard *et al.* 2005), and thus the presence of characteristics of interest.

Somatic embryogenesis is a tissue culture technique that is widely used in breeding and genetic studies of several plant species of economic interest (Kumaravel et al. 2017; Itaya et al. 2018; Zhu et al. 2018). Exploiting cellular totipotency, this technique is characterized by a high multiplication rate when compared with other propagation processes, plus it produces of plants identical to the mother plant, which is considered the best option for in vitro micropropagation. The development of diploid somatic cells in structures that match zygotic embryos depends on the balanced expression of a complex network of genes, which are regulated by endogenous and environmental factors (Zimmerman 1993). Therefore, identifying the genes involved in the process of somatic embryogenesis, and to understand their functions, is essential to improving their applicability in the process of plant propagation and breeding.

Different studies have been carried out in order to identify and characterize the genes regulating the onset of coffee somatic embryogenesis (Nic-Can *et al.* 2013; Silva *et al.* 2013; Silva *et al.* 2014; Torres *et al.* 2015; Freitas *et al.* 2017). Studies with several species have revealed the importance of such genes in the process of somatic embryogenesis (Wiśniewska *et al.* 2012; Silva *et al.* 2014; Tvorogova *et al.* 2016; Zheng *et al.* 2016; Boycheva *et al.* 2017). Likewise, research has shown that hormones and stress conditions influence the induction of this process (Mantiri *et al.* 2008b; Nolan *et al.* 2014), and induce the differentiation of cells leading to the onset of an embryogenic response (Ikeda-Iwai *et al.* 2003; Rose and Nolan 2006).

The genes belonging to the AP2/EREBP (APETALA2/ethylene-responsive element binding protein-protein that binds to the ethylene response element) superfamily are transcription factors that play important roles in plant development, as well as tolerance to biotic and biotic stress (Liu and Zhang 2017). Thus, signaling molecules, such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA), act to regulate several important defense-signaling pathways involving these genes (Bouaziz et al. 2015). A number of studies have attempted to identify and evaluate how these genes act in response to stress (Sharma et al. 2010; Bouaziz et al. 2015; Dossa et al. 2016; Du et al. 2016; Liu and Zhang 2017); however, few studies have aimed at identifying and characterizing these genes in somatic embryogenesis.

The AP2/EREBP superfamily is one of the largest groups of plant-specific transcription factors (Chen *et al.* 2016). This group shares a highly conserved region (AP2 DNA binding domain) with 50 to 70 amino acid residues (Allen *et al.* 1998). Based on the number of AP2 domains and similarity of sequences, genes belonging to the AP2/EREBP superfamily are divided into three families: AP2, RAV (related to ABI3/VP1), and ERF (ethylene response factor; Chen *et al.* 2016). The family AP2 possesses the AP2 domains (Shigyo and Ito, 2004), RAV possesses the AP2 domain and the B3 DNA binding domain (Feng *et al.* 2014; Li *et al.* 2015), and ERF contains a single AP2 domain, which is the largest of the AP2/EREBP superfamily and a crucial regulator of several processes in plants.

Different studies have shown the expression of ERF genes in different tissues and under different conditions, with these transcriptional factors being involved in several resposes to biotic and abiotic stresses in plants (Bouaziz et al. 2015; Dossa et al. 2016; Du et al. 2016; Liu and Zhang 2017). However, few studies have analyzed their role during the process of somatic embryogenesis (Mantiri et al. 2008b; Piyatrakul et al. 2012; Zheng et al. 2013; Silva et al. 2015). A gene belonging to the ERF subfamily, designated somatic embro-related factor 1 (SERF1), which is involved in somatic and zygotic embryogenesis and is induced by ethylene and is expressed in embryogenic calli, has been identified in Medicago truncatula (Mantiri et al. 2008b). This gene seems to be essential for somatic embryogenesis and may allow a connection between stress and plant development (Mantiri et al. 2008b; Nolan et al. 2014). The fact that the SERF1 gene belongs to the ERF subfamily and plays an important role in somatic embryogenesis is interesting since genes of this subfamily are commonly related to plant growth and processes of response to biotic and abiotic stressors (Bouaziz et al. 2015). This suggests, therefore, that other genes of this subfamily are likely to have important functions in the embryogenic process as well.

Currently, bioinformatics along with biotechnology allow the development of research aimed at genomic study. The transcriptome (Vieira *et al.* 2006) and, more recently, the genome sequencing (https://worldcoffeeresearch.org/) of *Coffea arabica* enabled the development of studies aiming the identification and analysis of genes related to the regulation of different metabolic pathways. In this context, gene expression analysis through real-time qPCR displays an important role for the understanding of the biological function of these genes. Therefore, considering the economic importance of the coffee and its cultivation problems, this study aimed to *in silico* identify and characterize the genes from the *Coffea arabica*. ERF

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subfamily analyzes the expression patterns of some of these genes during coffee somatic embryogenesis.

# **Materials and Methods**

*In Silico* Identification on the CAFEST Database The NCBI (National Center for Biotechnology) genebank was searched using *SERF1* as the keyword in order to find sequences relating to that gene already deposited in the database. The obtained sequences were annotated and applied in a new search using the BLAST (Basic Local Alignment Search Tool; Altschul *et al.* 1990) on the coffee EST (expressed sequence tag) database (CAFEST), which has been generated by the Coffee Brazilian Genome project (Vieira *et al.* 2006). The search and clustering processes of the of the putative members of the coffee ERF subfamily were carried out using the Gene Project interface (http://www.lge.ibi.unicamp.br/cafe/) from the CAFEST database.

In the same way, a second search was performed using the tBLASTn algorithm (Altschul *et al.* 1997). The *query* (target) of amino acids was compared with nucleotides translated from the database, and all *reads* that had significant alignment (e value > 10<sup>-5</sup>) were selected. Using the term *SERF1* as the keyword, a search of the ESTs database was performed, since all reads were previously annotated automatically by comparison with the NCBI genebank. Next, the selected reads were grouped (clustering), forming the EST-*conitgs* and the *singlets*. After clustering, the saturation process was performed aiming to find new sequences of the probable gene, as well as the correction of incomplete clusters. This process consisted of the application of the EST-*contigs* formed in the new search of the database. This procedure was repeated until no significant new reads were found.

Using the ORF FINDER (Open Reading Frame Finder) tool, we identified the open reading frame (ORF) from each gene obtained from the clustering process. The presence of the AP2 domain, which characterized ERF subfamily members, was verified through a conserved domain analysis carried out on the NCBI Conserved Domain database.

Finally, the nucleotide sequences of interest were compared with the public database of protein sequences (NCBI) using the BLASTx algorithm. The BLASTp algorithm (Altschul *et al.* 1997) was applied to align the protein of interest with the proteins deposited in NCBI, which allowed the evaluation of the degree of similarity between the compared sequences.

*In Silico* Identification on the Phytozome Database Using the keyword AP2, a search on the PFAM (https://pfam.xfam.org/) database, aiming to find the PFAM number for the AP2/EREBP superfamily, was carried out. The number obtained (PF00847) was used as a search parameter on the Phytozome



database (https://phytozome.jgi.doe.gov/pz/portal.html) for the obtaining the sequences from the ERF superfamily previously deposited on this database. The sequences found were then submitted to clustering using the CAP3 program and its default parameters. Similar to the sequences found on the CAFEST database, the ORF and the conserved ERF domain of the sequences found on the Phytozome database were identified through the ORF FINDER tool and verified on the NCBI Conserved Domain database, respectively.

Phylogenetic Analysis Using the ClustalW program (Thompson et al. 1994) and its standard parameters (default), a global alignment of the EST-contigs and singlets, obtained from the CAFEST and Phytozome databases, was performed with the amino acid sequences encoded by them, the SERF1 gene proteins of the species Medicago truncatula and Glycine max obtained from the NCBI, and the ERFs genes of Coffea arabica described by Lima et al. (2011). The sequences were visually inspected and manually corrected, with those whose homology could not be ascertained being removed. The final phylogenetic tree was constructed using the MEGA 7.0 program (Kumar et al. 2016), with a neighbor-joining comparison model (Saitou and Nei 1987), distance model p, and pairwise suppression. The probabilistic bootstrap test with 10,000 replicates was used to validate the tree in relation to the phylogenetic distance of the clusters (Sitnikova et al. 1995).

Identification of Common Grouping Motifs In order to identify conserved motifs from the deduced amino acid sequences of the among the putative *ERFs* found in the CAFEST and Phytozome databases, the MEME Suite (Version 4.11.3; http://meme-suite.org/tools/meme; Bailey *et al.* 2009) was employed with the following settings modified from the default: maximum number of motifs 6, minimum motif width 6, maximum motif width 200, and any number of repetitions. Sequences were then aligned using the ClustalW program (Thompson *et al.* 1994) and subjected to the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/) to shade the identical and similar amino acids in alignment.

**Electronic Northern** For *in silico* analysis of gene expression, the frequency of the reads forming EST-*contigs* and *singlets* was calculated according to their expression in the CAFEST libraries, and the data normalized to allow the exact analysis of the degree of expression of the probable genes in each of the treatments and organs of the plant. Normalization consisted of multiplying the number of reads forming each EST-*contig* or *singlet* by the ratio of the total number of all libraries and the number of reads in the library in which it appeared expressed. By relating genes and libraries, the data was dropped into an array and rearranged according to where they were expressed. The EST-*contigs, singlets*, and libraries were grouped by hierarchical clustering (Eisen *et al.* 1998) using the TreeView

program to obtain a figure that allows the degree of expression of the genes belonging to the ERF subfamily to be studied. The figure was generated in grayscale with zero or negative expression being represented by lighter shades, with gradually increasing darkness until reaching black, which represents the maximum degree of positive expression.

**Primer Design** From the candidate sequences for the genes involved in somatic embryogenesis belonging to the ERF subfamily of *Coffea arabica* obtained from the *in silico* analysis, primers for RT-qPCR (Table 1) were designed using Primer Express v3.0 of Applied Biosystems (Thermo Fisher Scientific, Foster City, CA).

Callus Induction and Establishment of Cellular Suspensions Embryogenic and non-embryogenic calli induction were generated from mother plants of cv. Catuaí Amarelo IAC 62 cultivated under greenhouse conditions using the protocol described by Teixeira et al. (2004). Initially, leaf explants with approximately 0.5 cm<sup>2</sup> were cultivated in primary medium (PM) for 30 d, and then, they were transferred to secondary medium (SM), where they were cultivated for 180 d. The PM medium is composed by 1/2-strength Murashige and Skoog salts (Murashige and Skoog 1962), sucrose (20 g  $L^{-1}$ ), thiamine  $(10 \text{ mg } \text{L}^{-1})$ , pyridoxine  $(1 \text{ mg } \text{L}^{-1})$ , glycine  $(1 \text{ mg } \text{L}^{-1})$ , nicotinic acid (1 mg  $L^{-1}$ ), myo-inositol (100 mg  $L^{-1}$ ), hydrolyzed casein (100 mg  $L^{-1}$ ), malt extract (400 mg  $L^{-1}$ ), 2,4-D (20.0 µM), IBA (4.92 µM), 2-iP (9.84 µM), and Phytagel®  $(2.4 \text{ g L}^{-1})$ . The SM medium constitution is basically the same of the PM medium, differing only by the 2,4-D concentration  $(10.0 \ \mu M)$ . The inoculated material was kept in a growth room at  $27 \pm 2^{\circ}$ C and absence of light.

Embryogenic cell suspensions were established with an inoculum density of 10 g L<sup>-1</sup> (Zamarripa *et al.* 1991) by 200 mg of embryogenic calli inoculation to 125-mL Erlenmeyer flasks containing 20 mL of T3 multiplication liquid medium: 1/2-strength Murashige and Skoog salts (Murashige and Skoog 1962), sucrose (30 g L<sup>-1</sup>), thiamine (5 mg L<sup>-1</sup>), pyridoxine (0.5 mg L<sup>-1</sup>), nicotinic acid (0.5 mg L<sup>-1</sup>), myo-inositol (100 mg L<sup>-1</sup>), hydrolyzed casein (100 mg L<sup>-1</sup>), malt extract (200 mg L<sup>-1</sup>), kinetin (4.44  $\mu$ M), and 2,4-D (4.52  $\mu$ M) (vanBoxtel and Berthouly 1996). Flasks were maintained in an orbital agitator at 100 rpm (Tecnal, Piracicaba, Brazil), at 25 ± 2°C and in the absence of light, and the multiplication medium was completely renewed at every 15 d. After 90 d, the embryogenic cell suspensions were used in the experiment.

**Experimental Design and Tissue Sampling** The experiment was conducted in a completely randomized design, with three biological repetitions for each tissue type. For non-embryogenic and embryogenic callus cultures, each repetition was composed by 10 calluses generated from different leaf explants. For cell suspensions, each biological repetition was composed by 200 mg of cell clusters obtained after embryogeneic calli multiplication in liquid medium. Collected samples were immediately frozen in liquid nitrogen and subsequently stored at  $- 80^{\circ}$ C until RNA extraction.

Extraction of Total RNA and cDNA Synthesis The extraction of RNA from the samples was performed using Concert<sup>TM</sup> Plant RNA Reagent extractor reagent (Invitrogen Corporation, Carlsbad, CA). The samples were then treated with the Turbo DNA-Free Kit (Applied Biosystems–Thermo Fisher Scientific, Vilnius, Lithuania) for total removal of genomic DNA. The quantity and purity of the RNA were measured with a ND-1000 Nanodrop® Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The High-Capacity cDNA Reverse Transcription Kit (Invitrogen–Thermo Fisher Scientific, Vilnius, Lithuania) was used for the synthesis of cDNA from 1000 ng of RNA.

**RT-qPCR** Analysis of gene expression by RT-qPCR employed ABI PRISM 7500 Real-Time PCR (Applied Biosystems– Thermo Fisher, Singapore), using SYBR Green (Applied Biosystems–Thermo Fisher Scientific). The cDNA was obtained from RNA extracted from the three tissues under study: nonembryogenic callus, embryogenic callus, and embryogenic cell suspension.

Gene	GenBank accession number	Primers (5'-3')	Amplification efficiency (%)
RPL39 ribosomal	GT720707.1	Fw: GCGAAGAAGCAGAGGCAGAA	87
protein L39		Rv: TTGGCATTGTAGCGGATGGT	
24S ribosomal	GT730897.1	Fw: GACCAATCGTCTTCTTTCCAGAAA	100
protein 24S		Rv: TCAACTCAGCCTTGGAAACATTAG	
ERF8 (Contig6)	AHA93902.1	Fw: AGCAGCAAGTCCTTCCCAAG	98
		Rv: AATCTCAGCAGCCCATTTCC	
ERF12 (Contig27)	AHA93911.1	Fw: CCTCCAACGCTTCTTTCAGC	90
		Rv: CGGTTTGTCTTGGGTGGTTT	
ERF13 (Contig9)	AHA93904.1	Fw: GCTGGGATTGGCTTCATTTG	100
		Rv: ACCGAGAAAGTTGCTGCGTA	

**Table 1.** Sequence of *primers*used in RT-qPCR. *Fw* forwardprimer and *Rv* reverse primer



The thermal conditions of the reaction were 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. Data were collected and stored in the program 7500 Fast Software (Version 2.3; Applied Biosystems-Thermo Fisher). Each reaction used 1 µL of cDNA (10 ng/  $\mu$ L), 0.2  $\mu$ L of each primer (initial concentration of 10  $\mu$ M), and 5.0 µL of Master Mix SYBR green UDG with ROX (Applied Biosystems-Thermo Fisher) for a final volume of 10.0 µL per sample. The samples were processed in technical triplicate. The results were normalized using Cq (quantification cycle), also known as the threshold cycle (Ct), obtained by the expression of the reference genes of ribosomal protein L39 (RPL39) and ribosomal protein 24S (24S; Freitas et al. 2017) present in the same reaction. The Cq was determined by the number of cycles in which the fluorescence generated within a reaction crossed the threshold line. The Pfaffl formula was used to calculate the relative expression (Pfaffl 2001).

Normalization was performed using the equation  $\Delta CT_{target} = CT$  (target gene) – CT (endogenous control) and  $\Delta CT_{reference} = CT$  (target gene) – CT (endogenous control). The calibration was determined by the formula  $E_{target} \stackrel{\Delta CTtarget}{=} and E_{reference} \stackrel{\Delta CTreference}{=}, where E is the efficiency value of the primers used. The calibrator was a sample used as the basis for comparative expression results. The relative quantification was obtained by the formula <math>E_{target} \stackrel{\Delta CTreference}{=} (Pfaffl 2001).$ 

# Results

Analysis In Silico – CAFEST Database The search for sequences forming the genes belonging to the ERF subfamily in CAFEST resulted in 179 reads, forming 30 EST-contigs and 37 singlets after the clustering process. Next, 27 EST-contigs and 21 singlets were selected through the conserved AP2 characteristic of the ERF/DREB family (Nakano et al. 2006). However, after analysis of the amino acid residues of the AP2 domain (Fig. 1), 15 EST-contigs and 5 singlets having the specific ammino acids of the ERF subfamily (Sakuma et al. 2002; Liu and Zhang 2017) remained; the sequences that did not show the specific amino acid residues of the ERF subfamily were excluded from the analyses.

**Phytozome Database** The search for sequences forming the genes belonging to the ERF subfamily in Phytozome database resulted in 254 reads, forming 103 *Phy-contigs* and 39 *Phy-singlets* after the clustering process. Then, based on the on the presence of the AP2 conserved domain, 108 sequences were selected. However, after analyzing the amino acid residues of the AP2 domain (Fig. 2), only 54 sequences, 35 *Phy-contigs* and 19 *Phy-singlets*, were found to display the specific amino acid residues of the ERF subfamily (Sakuma *et al.* 2002; Liu and Zhang 2017). The sequences that did not show the



specific amino acid residues of the ERF subfamily were excluded from the analyses.

**Phylogenetic Analysis** The 15 EST-*contigs* and 5 *singlets* obtained from the CAFEST and the 35 *Phy-contigs* and 19 *Physinglets* obtained from the Phytozome databases were submitted to phylogenetic analysis along with the *SERF1* gene from *Medicago truncatula* and *Glycine max* and the *ERFs* gene of *Coffea arabica* described by Lima *et al.* (2011), with the aim of analyzing the phylogenetic distances among the studied sequences (Fig. 3).

Figure 3 shows that the EST-contigs2, 4, 6, 7, 9, 10, 12, 17, 20, 21, 26, and 27 and the singlet36, along with Phy-contig99, Phy-contig92, Phy-singlet4, Phy-contig37, Phy-singlet12, Phy-contig50, Phy-contig77, Phy-contig101, Phy-contig93, Phy-singlet27, Phy-contig98, and Phy-contig47, grouped with the genes CaERF10, CaERF2, CaERF8, CaERF1, CaERF13, CaERF5, CaERF3, CaERF6, CaERF9, CaERF4, CaERF11, CaERF12, and CaERF15, respectively, forming independent clades with bootstrap values above 99. The gene SERF1 from Medicago truncatula and Glycine max formed a well-defined clade, grouping with Phy-contig97. However, none of the EST-contigs and singlets evaluated exhibited a high degree of similarity with these sequences, so they were not grouped with them.

The EST-contigs and singlets and Phy-contigs and Physinglets that clustered close to the CaERFs genes were submitted to alignment thorugh the ClustalW program (Thompson et al. 1994). The result obtained showed a high similarity among the sequences, allowing the observation of small differences in sequence length and identity (Online Resource 1). Considering the high similarity between the sequences found in the CAFEST and Phytozome databases, and the possibility of analyzing the in silico expression profile of the CAFEST sequences, through the Electronic Northern, only the EST-contigs and singlets were submitted to the aligment on the NCBI database through the Blastx tool (Table 2) for the comparison of the identity level of these sequences with those present in this database. As one can observe in Table 2, all sequences that grouped close to CaERF genes showed identity levels above 98%. In addition, CAFEST sequences allow the in silico expression analysis to be carried out through the Electronic Northern.

**Expression Analysis by Electronic Northern** Gene expression analysis by means of Electronic Northern made it possible to gauge the expression levels of the EST-*contigs* and *singlets* in different tissues, thus allowing the prediction of probable sequences for the genes of interest (Fig. 4). The Electronic Northern consists of counting the reads that form each EST-*contig* and *singlet*, indicating the degree of expression of each sequence in the tissue of interest.

Figure 4 shows that EST-*contig6* was expressed in the tissues of the embryogenic calluses (EA1/IA1/IA2) and cell

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	Contig2	:	FRGVRQRPWGKFA	AEIRD	PSKNGARVWLGTYETAEEAALAYDRAAYRMRGSKALLNFP	:	58
	Contig4	:	NYRGVRQRPWGKW	AAEIR	DPRKAARVWLGTFETAEDAARAYDRAAIEFRGPRAKLNFP	:	58
	Contig6	:	LYRGIRQRPWGKW	AAEIR	DPRKGVRVWLGTFNTAEEAARAYDKEARKIRGKKAKVNFP	:	58
	Contig7	:	KYRGIRQRPWGKW	AAEIR	DPQKGVRVWLGTFNTAEEAARAYDKAAKRIRGDKAKLNFV	:	58
	Contig9	:	YRGVRRRPWGKYA	AEIRD	STRNGTRVWLGTFQTAEEAALAYDRAAFRMRGAKALLNFP	:	58
	Contig10	:	QYRGIRQRPWGKW	AAEIR	DPRKGVRVWLGTFNTAEEAARAYDTEARRIRGKKAKVNFP	:	58
	Contig12	:	HYRGIRQRPWGKW	AAEIR	DPRKGVRVWLGTFNTAEAAARAYDTEARRIRGKKAKLNFP	:	58
	Contig15	:	HFRGVRKRPWGRY	AAEIR	DPSKKSRVWLGTFDTAEEAARAYDAAAREFRGPKAKTNFP	:	58
	Contig17	:	HFRGVRKRPWGRY	AAEIR	DPGKKSRVWLGTFDTAEEAARAYDAAARDFRGPKAKTNFP	:	58
	Contig20	:	RFRGVRKRPWGRF	AAEIR	DPWKKTRVWLGTFDSAEDAARAYDAAARTLRGPKAKTNFP	:	58
	Contig21	:	HFRGVRKRPWGRY	AAEIR	DPWKKTRRWLGTFDTAEEAALAYDEAARSLRGPKAKTNFG	:	58
	Contig22	:	KYRGIRQRPWGKW	AAEIR	DPQKGVRVWLGTFNTAEEAARAYDKAAKRIRGDKAKLNFV	:	58
	Contig26	:	RYRGVRQRPWGKW	AAEIR	DPHKAARVWLGTFDTAEAAARAYDEAALRFRGNRAKLNFP	:	58
	Contig27	:	YRGVRQRPWGKFA	AEIRD	PNRKGSRVWLGTFDTAVEAAKAYDKAAFRLRGSKAILNFP	:	58
	Contig28	:	HYRGVRQRPWGKW	AAEIR	DPKKAARVWLGTFETAEAAALAYDEAALRFKGNKAKLNFP	:	58
	Singlet10	:	KFRGVRQRPWGRW	AAEIR	DPTWKKRVWLGTFDTPEEAASAYDRAAVKLKGPDAVTNFP	:	58
	Singlet21	:	FRGVRQRPWGKFA	AEIRD	PAKNGARVWLGTYETAXXAALAYDRAAYXMRGXKALLNSL	:	58
	Singlet30	:	HYRGIRQRPWGKW	AAEIR	DPRKGVRVWLGTFNTAEGAARAYDTEARRIRGKKAKLNFP	:	58
	Singlet34	:	HYRGIRQRPWGKW	AAEIR	RPRKGVRVWLGTFNTGEAAGRAYDTEARRIRRKKGNLNFP	:	58
	Singlet36	:	HYRGVRQRPWGKW	AAEIR	DPKKAARVWLGTFETAEAAALAYDEAALRFKGNKAKLNFP	:	58
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	Contig5	:	NYRGVRQRTWGKW	VAEIR	PKKGSRLWLGTFGTAIEAALAYDKAARVMYGPSAQLNLP	:	58
	Contig8	:	NYRGVRQRTWGKW	VAEIR	PNRGSRLWLGTFGTAVEAALAYDEAARSMYGPCARLNLP	:	58
	Contig11	:	RGIRRRRWNSNKW	VCELR	PNKQSRIWLGTYPTAEMAARAYDVAALALRGHLACLNFA	:	58
	Contig16	:	LYRGVRQRHWGKW	VAEIR	LPKNRTRLWLGTFDTAEEAALAYDKAAFKLRGDFTRLNFP	:	58
	Contig18	:	VYRGVRMRAWGKW	VSEIR	PKKKSRIWLGTFACPEMAARAHDVAALSIKGNSAILNFP	:	58
	Contig23	:	LYKGIRMRKWGKW	VAEIR	PNKRSRIWLGSYSTPVAAARAYDTAVYYLRGPTARLNFP	:	58
	Contig24	:	TYRGVRQRTWGKW	VAEIR	PNHGARVWLGTFNTSYEAARAYDDAAKRLYGKCAKLNLP	:	58
	Contig30	:	HFRGVRSR-SGKW	VSEIR	PRKTTRIWLGTYPTPEMAAAAYDAATLALKGPDAILNFP	:	57
	Singlet2	:	RYRGVRQRHWGSW	VSEIR.	HPILKTRIWLGTFETAEDAARAYDEAARLMCGPRARSNFP	:	58
	Singlet3	:	AYKGVRQRTWGKW	VAEIR	PNRGSRLWLGTFDTSHEAAIAYDAAARKLYGTAAKLNLP	:	58
	Singlet6	:	KFRGVRQRHWGSW	VSEIR.	HPLLKRRVWLGTFDTAEEAARAYDQAAVLMSGRNAKTNFP	:	58
	Singlet8	:	LYRGVRQRHWGKW	VAEIR	LPKNRTRLWLGTFDTAEGAALAYDKAAFKLRSDFTRAELP	:	58
	Singlet11	:	TYHGVRRRSWGKW	VSEIR	PRKKSRIWLGTFATPEMAARAHDVAAIAIKGHSAFLNFP	:	58
	Singlet31	:	LYRGVRQRHWGKW	VAEIR	LPKNRTRLWLGTFDTAEEAALAYDKAAYTLRGEFARLNFP	:	58
	Singlet35	:	KFVGVRQRPSGKW	VAEIK	NTTHKIRMWLGTFDTAEETAQAYDEAACLLRGSNTRTNFM	:	58
	Singlet37	:	LYRGVRMRNWGKW	VSEIR	PRKKSRIWLGTFPSPEMAARAHDVAALSIKGTSAILNFP	:	58

Figure 1. Conserved amino acid residues (shaded in *black*) of the AP2 domain from EST-*contigs* and *singlets* that characterize the ERF (*a*) and DREB (*b*) subfamilies.

suspensions treated with acibenzolar-S-methyl and brassinosteroids (CB1); however, its greatest expression was in embryogenic calluses. The EST-*contig9*, as well as EST-*contig6*, was expressed in the libraries of embryogenic calluses and cells in suspension treated with acibenzolar-S-methyl and brassinosteroids (CB1), but a greater expression was observed in the library of the hypocotyls treated with acibenzolar-S-methyl (CL2; Fig. 4). The expression profile of EST-*contig17* (Fig. 4) was varied, with expression in five tissues: cells in suspension treated with NaCl (CS1), embryogenic calli (EA1/IA1/IA2), flower buds in different stages of flowering (FB1/FB2/FB4), floral buds and fruits of different stages (FR1/FR2), and field plants with stress from water deficit (tissue pool; SH2). The EST-*contig20* was expressed in

cells in suspension treated with acibenzolar-S-methyl and brassinosteroids (CB1), and young leaves of orthotropic branches (LV4/LV5; Fig. 4). Similar to EST-*contig6*, 9, and 20, the sequence of EST-*contig27* was expressed in the tissue of cells in suspension treated with acibenzolar-S-methyl and brassinosteroids (CB1); however, the expression of this EST-*contig* was also observed in the germinating seed library (whole seeds and zygotic embryos; EM1, SI3; Fig. 4). The expression of EST-*contig28* was observed in both embryogenic callus (EA1/IA1/IA2) and non-embryogenic callus (CA1/IC1/PC1; Fig. 4). The *singlets 10, 21*, and *36* were expressed in cells in suspension treated with acibenzolar-S-methyl (BP1), cells in suspension treated with NaC1 (CS1), and embryogenic calli (EA1/IA1/IA2), respectively (Fig. 4).



а			1	b			
Phy-Contig8 :	YRGVRRRPWGKYA	EIR STRKGIRVWLGTFDSAEAAALAYDQAAFCMRGPMAVLNFP	: 58	Dhu Contini	. KYKCUDKDKNCKN	CETEL DUCDED THE COURSERS 3D 3D 2D 3T ECL DOWNS VENDS	
Phy-Contig13 :	YRGVRKRPWGKYA	EIROMNNGARLWLGTFATAEEAARAYDRAAFATRGSLAVLNFP	: 58	Phy=Contig1	: KINGVRARAWGAW	SETREPNSKEKTWEGSTUSAEKAAKAFDAALFCERGKNAKFNFA	: 50
Phy-Contig15 :	KYRGVRQRPWGKW	AEIRDPARKARIWLGTYDTAEEAAMVYDRAAIQIRGPDALTNFI	: 58	Phy=Contig2	: DQSKVRQRSAGKW	ALIRDPROGROVWLGIFISALLARAIDRACIOPRODE KODRAKINFF	: 50
Phy-Contig22 :	YIGVRKRPWGKYA	EIRDSTRNGMRVWLGTFESPEEAALAYDQAAFSMRGPLASLNFP	: 58	Phy-Contigs	: QSKRVRQKSAGKW	AETRUPHUGRSVWLGTFTSAEEAARATURKCTUFRGURAKTNFP	: 58
Phy-Contig23 :	YVGVRKRPWGKFA	EIRDSTRNGIRVWLGTFDTAEEAALAYDQAALSMRGPVAALNFP	: 58	Phy-Contigs	: KFKGIKKKKWGKW	SEIRVPGIQDRLWLGSIAAPEAAAMAHDIAIICLKENASLDDFN	: 50
Phy-Contig24 :	YIGVRKRPWGKYA	EIRDSTRNGIRVWLGTFDRPEQAALAYDQAAFSMRGTLASLNFP	: 58	Phy-Contig6	: KFVGVRQRPSGRW	AEIKNTTQKIRMWLGTFDTAEEAAQAYDEAACLLRGSNTRTNFM	: 58
Phy-Contig25 :	YIGVRKRPWGKYA	EIRDSTRNGLRVWLGTFDSAEEAALAYDOAALAMRGPATALNFP	: 58	Phy-Contig16	: KFRGVRQRCWGSW	AEIRDPIKQKQVWLGTIDTAEKAAIAIDKAAIQFRGPKAITNFL	: 58
Phy-Contig30 :	YRGVRRRPWGKYA	EIRDSTRNGVRVWIGTFDTAEEAALVYDQAAFALRGHAAVLNFP	: 58	Phy-Contig19	: PYRGIRMRKWGKW	AEIRDPNKRSRIWLGSYSSPVAAARAYDTAVFYLRGPSARLNFP	: 58
Phy-Contig34 :	YRGVRRRPWGKFA	EIRNPNKKGSRIWLGTYETPEDAAMAYDLAAFEIRGARALLNFP	: 58	Phy-Contig20	: AYKGVRQRTWGKW	AEIRDPNRGSRLWLGTFDTSHEAAIAYDAAARKLYGTAAKLNLP	: 58
Phy-Contig35 :	YRGVRRRPWGTYA	EIRDPKKKGSRIWLGTYETPEDAALAFDOAAFKMRGARARVNFP	: 58	Phy-Contig21	: TIKGVRORTWGKW	AEIRPNCGSRLWLGTFDTSHEAAIAIDAAAIRLIGEAARLNLP	: 58
Phy-Contig37 :	KYRGIRORPWGKW	AEIRDPOKGVRVWLGTFNTAEEAARAYDKAAKRIRGDKAKLNFV	: 58	Phy-Contig28	: IRGVRRRRSSGRW	SEIRPRTPNRIWLGTFPTPEMAAAAIDVAALALKGPEAELNFP	: 58
Phy-Contig38 :	RFLGVRRRPWGRY	AEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGTQARTNFV	: 58	Phy-Contig29	: LYRGVRMRNWGRW	SEIRDPRKKSRIWLGTFPSPEMAARAHDVAALSIKGTSAILNFP	: 58
Phy-Contig40 :	YRGVRRRPWGKYA	EIRDPTRQGARLWLGTFETAEEAARAYDKAAFNLRGHLAILNFP	: 58	Phy-Contig31	: NYRGVRQRTWGRW	AEIROPNKGSKLWLGTFGTAVEAALAYDEAAKSMYGPCAKLNLP	: 58
Phy-Contig41 :	YRGVRRRPWGKFA	EIRDSTRNGIRVWLGTFDSAEAAALAYDOAAFSMRGOAAILNFP	: 58	Phy-Contig32	: LIRGVRORHWGRW	AEIRLPKNRTRLWLGTFDTAEEAALAIDKAAIKLRGEFARLNFP	: 58
Phy-Contig45 :	NYRGVRKRPWGRW	SAEIRDRIGRCRHWLGTFDTAEEAARAYDAAARKLRGSKARTNFO	: 58	Phy-Contig33	: TYRGVRKRNWGKW	SEIRBPRKKSRIWLGTYPTAEMAARAHDVAALAIKGHSAYLNFP	: 58
Phy-Contig47 :	HYRGVRORPWGKW	AEIRDPKKAARVWLGTFETAEAAALAYDEAALRFKGNKAKLNFP	: 58	Phy-Contig42	: LFRGVRQRHWGRW	AEIRLPRNKTRVWLGTFDTAEEAAFAYDTAAYMLRGDYAHMNFP	: 58
Phy-Contig48 :	YIGVRKRPWGKYA	EIRDSTRNGMRVWLGTFATAEEAALAYDOAALSVRGPTTALNFP	: 58	Phy-Contig43	: VIRGVRMRSWGKW	SEIRBPRKKSRIWLGTISTAEMAARAHDVAALSIKGNSAILNFP	: 58
Phy-Contig50 :	HYRGIRORPWGKW	AEIRDPRKGVRVWLGTFNTAEGAARAYDTEARRIRGKKAKLNFP	: 58	Phy-Contig44	: KFRGVRQRHWGSW	SEIRHPLLKRRVWLGTFETAEEAARAYDEAAVLMSGRNAKTNFP	: 58
Phy-Contig55 :	YRGVRRRPWGKFA	EIR PAKNGARVWLGTFETAEDAAIAYDRAAYRMRGARALLNFP	: 58	Phy-Contig46	: RYRGVRQRHWGSW	SEIRHPLLKTRIWLGTFETAEDAARAYDEAARLMCGPRARTNFA	: 58
Phy-Contig56 :	YRGVRORPWGKYA	EIROPKRRGSRVWLGTFDTAIEAAKAYDRAAFKMRGSKAILNFP	: 58	Phy-Contig49	: RFVGVRQRPSGRW	AEIKDSSQRIRLWLGTYDTPEEAARAYDEAARALRGENARTNFA	: 58
Phy-Contig70 :	HFRGVRKRPWGRY	AEIRDPGKKSRVWLGTFDTAEEAARAYDAAAREFRGAKAKTNFP	: 58	Phy-Contig52	: EYRGVRQRTWGKW	AEIRMPKKRTRLWLGSFATAEEAAMAYDEAARRLYGPDAYVNLP	: 58
Phy-Contig71 :	HYRGVRKRPWGRY	AEIRDPWKKTRVWLGTFDTPEEAALAYDGAARSLRGAKAKTNFP	: 58	Phy-Contig54	: LYRGVRQRHWGKW	AEIRLPONRMRVWLGTYDTAEAAAYAYDRAAYKLRGEYARLNFP	: 58
Phy-Contig73 :	YRGVRRRPWGKFA	EIROPKKKGSRIWLGTYETPEEAAMAYDRAAFEIRGARAMLNFP	: 58	Phy-Contig5/	: TYHGVRRRSWGKW	SEIRDPRKKSRIWLGTFATPEMAARAHDVAAIAIKGHSAFLNFP	: 58
Phy-Contig76 :	RYRGVRRRPWGRY	AEIRDPOSKERRWLGTFDTAEEAACAYDCAARAMRGVKARTNFV	: 58	Phy-Contig58	: RGIRRRRWNSNKW	CELREPNKQSRIWLGTYPTAEMAARAYDVAALALRGHLACLNFA	: 58
Phy-Contig77 :	HFRGVRKRPWGRY	AEIRDPSKKSRVWLGTFDTAEEAARAYDAAAREFRGPKAKTNFP	: 58	Phy-Contig59	: KYKGVRMRSWGSW	SEIRAPNQKTRIWLGSYSTAEAAARAYDAALLCLKGSSANLNFP	: 58
Phy-Contig84 :	YRGVRRRPWGKYA	EIRSTRNGTRVWLGTFOTAEEAALAYDRAAFRMRGAKALLNFP	: 58	Phy-Contig60	: KYVGVRQRPSGRW	AEIRDTTOKIRMWLGTFETAEEAARAYDEAACLLRGSNTRTNFV	: 58
Phy-Contig85	YRGVRRRPWGKYA	EIRDSSRKGARLWLGTFGTAEEAAMAYDKAALKIRGPKAYLNFP	: 58	Phy-Contig61	: SYIGIRQRKWGKW	SEIRPGKKSRIWLGSYEAPELAAAAYDVAALHLKGARARLNFP	: 58
Phy-Contig87	RYRGVRRRPWGKW	AEIRDPYKAARVWLGTFDTAEAAARAYDEAALRFRGNKAKLNFP	: 58	Phy-Contig62	: LYRGVRQRHWGKW	AEIRLPKNRTRLWLGTFDTAEEAALAYDKAAFKLRGDFTRLNFP	: 58
Phy-Contig92	NYRGVRORPWGKW	AETROPRKAARVWLGTFETAEDAARAYDRAATEFRGPRAKLNFP	: 58	Phy-Contig63	: TYRGVRQRTWGKW	AEIROPNHGARVWLGTFNTSYEAARAYDDAAKRLYGKCAKLNLP	: 58
Phy-Contig93	HFRGVRKRPWGRY	AEIRDPWKKTRRWLGTFDTAEEAALAYDEAARSLRGPKAKTNFG	: 58	Phy-Contig65	: VYRGVRMRAWGKW	SEIROPKKKSRIWLGTFACPEMAARAHDVAALSIKGNSAILNFP	: 58
Phy-Contig96	KFRGVRORPWGKW	AEIRDPSTRARLWLGTYDTAEEAAMVYDNAAIKLRGPDALTNFI	: 58	Phy-Contig66	: KFRGVRQRHWGSW	SEIRHPLLKRRVWLGTFDTAEEAARAYDQAAVLMSGRNAKTNFP	: 58
Phy-Contig97 :	YRGVRRRPWGKYA	EIRMPVRKGSRVWLGTFDTAMDAAKAYDSAAFKMRGRKAILNFP	: 58	Phy-Contig68	: RYVGVRQRPSGRW	AEIKDSLQKVRLWLGTFDTAEDAARAYDEAARALRGANARTNFE	: 58
Phy-Contig98	YRGVRORPWGKFA	AFIR PNRKGSRVWLGTFDTAVEAAKAYDKAAFRLRGSKAILNFP	: 58	Phy-Contig69	: RFLGVRQRPSGRW	AEIKDSSQKLRLWLGTFDTAEEAALAYDNAARLLRGRNAKTNFT	: 58
Phy-Contig99 :	FRGVRORPWGKFA	EIR PAKNGARVWLGTYETAEEAALAYDRAAYRMRGSKALLNFP	: 58	Phy-Contig/2	: HFRGVRQRSEGRW	AEIRNPHLGKTEWLGTFTSAEEAARAFDRKCIQIKGDRAKTNFP	: 58
Phy-Contig101 :	RFRGVRKRPWGRF	AEIRDPWKKTRVWLGTFDSAEDAARAYDAAARTLRGPKAKTNFP	: 58	Phy-Contig/8	: LIRGVRORHWGRW	AEIRLPRNKTRLWLGTFDTAEDAALAIDREAFKLRGENARLNFP	: 58
Phy-Singlet3 :	YRGIRRRPWGKYA	EIRPTRNGARLWLGTFDTAEEAARAYDRAAYALRGHOALLNFP	: 58	Phy-Contig/9	: RIRGVRQRHWGSW	SEIRHPILKTRIWLGTFETAEDAARAYDEAARLMCGPRARSNFP	: 58
Phy-Singlet4 :	LYRGIRORPWGKW	AEIRDPRKGVRVWLGTFNTAEEAARAYDKEARKIRGKKAKVNFP	: 58	Phy-Contig82	: LIKGIRMKKWGKW	AEIR PNKRSKIWLGSISTPVAARAIDTAVIILRGPTARLNFP	: 58
Phy-Singlet5 :	HYRGVRORPWGKW	AEIRDPKKPARVWLGTFETAEAAALAYDEAALRFEGNKAKLNFP	: 58	Phy-Contigse	: NIRGVRORIWGRW	AEIRPERKGSRLWLGTFGTAIEAALAIDKAARVMIGPSAQLNLP	: 58
Phy-Singlet6 :	KFRGVRORPWGRW	AEIRDPTKRKRVWLGTYDTPEEAASVYDRAAVKLKGPDAVTNFP	: 58	Phy-Contig88	: PMIRGIRSRSGRW	SEIREPRETERIWLGTIPTPEMAAAAIDVAAIALKGSDIALNFP	: 58
Phy-Singlet8	YIGVRKRPWGKYA	EIRSTRNGMRVWLGTFATAEEAALAYDOAALSVRGPTTALNFP	: 58	Phy-Contig90	: KYTGVRKRKWRKY	SEIRLPNSRERIWLGSYDTAEKAARAFDAALFCLRGKSAKFNFP	: 58
Phy-Singlet9 :	YIGVRKRPWGKYA	EIR STRHGLRVWLGTFDSPEEAAFAYDOAAFSLRGPMASLNFP	: 58	Phy-Contig94	: VIRGIRRENSGEW	CEVREPNKKSKIWLGTFPTAEMAAKAHDVAAIALKGKSACLNFA	: 58
Phy-Singlet12 :	OYRGIRORPWGKW	AEIRDPRKGVRVWLGTFNTAEEAARAYDTEARRIRGKKAKVNFP	: 58	Phy-Contig95	: PHFRGVRSRSGRW	SEIR PRKTTRIWLGTIPTPEMAAAAIDAATLALKGPDAILNFP	: 58
Phy-Singlet17 :	QYRGIRQRPWGKW	AEIRDPRKGVRVWLGTFNTAEEAARAYDTEARRIRGKKAKVNFP	: 58	Phy-Contigiou	. INDEVDODUNERN	ALIREPRESERVED IN CORPORATEDAARAIDRAAIILIGSRAULNEU	: 58
Phy-Singlet18 :	KFRGVRORPWGKW	AEIRDPSRRVRLWLGTYDTAEEAAMVYDHAAIOLRGPDALTNFA	: 58	Phy-Contigius	S IIRGVRQRHWGRW	AEIRLPRNKKKLWLGTFDTAEEAALAIDREAFKLRGENAKLNFP	: 58
Phy-Singlet19 :	RYLGVRRROWGRY	AEIRNPYTKERHWLGTFDTAEEAAVAYDLASISFSGIOKARTNF	: 58	Phy-Singlet/	: VIRGVRKRRWGRW	SETREPGKKTKIWLGSFDTEEMAAVAHDAAAIFFRGSAAQLNFP	: 58
Phy-Singlet21 :	HYRGVRRRPWGKW	AEIRDPKKAARVWLGTFDMAEDAALAYDEAALRFKGTKAKLNFP	: 58	Phy-Singlet24	KFRGVRQRQWGSW	SEIRHPLEKKRIWLGIFEIAEAAARAIDQAAILMNGQNAKINFP	: 58
Phy-Singlet22 :	RFLGVRRRPWGRY	AEIRDPTTKERHWLGTFDTAEEAALAYDRAARSMRGSRARTNFV	: 58	Fny=Singiet25	. AFRGVRQRQWGSW	SEIKHFBBKKKIWEGIFEIAEAAAKAIDEAAIEMNGQNAKTNFP	: 58
Phy-Singlet23 :	RFLGVRRRPWGRY	AEIRDPTTKERHWLGTFDTAEEAALAYDRAARSMRGSRARTNFV	: 58	Phy-Singlet26	: KEVGVRQRPSGRW	AEIKDTIQKIKVWLGTFDTAEEAARAYDEAACLLRGANTRTNFW	: 58
Phy-Singlet27 :	RYRGVRQRPWGKW	AEIRDPHKAARVWLGTFDTAEAAARAYDEAALRFRGNRAKLNFP	: 58	rhy-Singlet33	: KIKGVRMRKWGKW	AEVKUPKSKUKIWLGSIQTAEEAAKAIDAAAFCLRGPSVMLNFP	: 58
Phy-Singlet28 :	KFLGVRRRPWGRF	AEIRDPTTKERHWLGTFDTAEEAALAYDKAARSMRGARARTNFV	: 58	rny-Singlet38	S : KIKGVRMRKWGKW	ALVKUPKSRUKIWLGSIUTAEEAARAIDAAAFCLRGPSVMLNFP	: 58
Phy-Singlet29 :	KFLGVRRRPWGRF	AEIRDPTTKERHWLGTFDTAEEAALAYDKAARSMRGSRARTNFV	: 58	rny-Singlet39	<ul> <li>REHGVRQWRWGSW</li> </ul>	ABIGDEVNQNQVWLGTIDTTEEAAMEIDHAAIRFRGPKAITNFP	: 58
Phy-Singlet30 :	RYRGVRORPWGKW	AEIRDPHKAARVWLGTFDTAEAAARAYDEAALRFRGNRAKLNFP	: 58				
Phy-Singlet34 :	YRGIRRRPWGKYA	EIRDPTRNGARLWLGTFDTAEEAARAYDRAAYALRGHOALLNFP	: 58				
Phy-Singlet35 :	HYRGVRQRPWGKW	AEIRDPKKPARVWLGTFKTAEAAALAYDEAALRFEGNKAKLNFP	: 58				

Figure 2. Conserved amino acid residues (shaded in *black*) of the AP2 domain from *Phy-contigs* and *Phy-singlets* that characterize the ERF (*a*) and DREB (*b*) subfamilies.



**Figure 3.** Phylogenetic tree of all sequences (ETS-*contigs* and *singlet*, *Phy-contigs*, and *Phy-singlets*) belonging to the ERF subfamily, *SERF1* gene of *Medicago truncatula* Gaertn., and *ERFs* gene of *Coffea arabica* L. (*circles*) EST-*contigs* and *singlets*, (*diamonds*) *Phy-contigs* e *Phy*-

*singlets*, (*squares*) *ERFs* gene of coffee *Coffea arabica*, (*triangles*) genes *MtSERF1*, *GmSERF1*, and *GmSERF2*. The *neighbor-joining* algorithm was used to construct the tree by the method of *p-distance* and *pair-wise* suppression.



Subfamily	Contig/ aa	blastx	e value	Identity	Positives
ERF	CaC2	AHA93900.1 ethylene response factor 10 [Coffea arabica L.]. 264aa	$1e^{-142}$	264/264 (100%)	264/264 (100%)
	CaC4	AHA93901.1 ethylene response factor 2 [Coffea arabica L.]. 304aa	$9e^{-133}$	304/304(100%)	304/304(100%)
	CaC6	AHA93902.1 ethylene response factor 8, partial [Coffea arabica L.]. 249aa	$1e^{-130}$	177/178 (99%)	177/178 (99%)
	CaC7	AHA93903.1 ethylene response factor 1 [Coffea arabica L.]. 218aa	$5e^{-117}$	218/218(100%)	218/218(100%)
	CaC9	AHA93904.1 ethylene response factor 13 [Coffea arabica L.]. 193aa	$9e^{-119}$	193/193 (100%)	193/193 (100%)
	CaC10	AHA93905.1 ethylene response factor 5 [Coffea arabica L.]. 396aa	0.0	388/396(98%)	389/396(98%)
	CaC12	AHA93906.1 ethylene response factor 3, partial [Coffea arabica L.]. 335aa	0.0	335/335(100%)	335/335(100%)
	CaC17	AHA93907.1 ethylene response factor 6, partial [Coffea arabica L.]. 220aa	$4e^{-97}$	220/220 (100%)	220/220 (100%)
	CaC20	AHA93908.1 ethylene response factor 9, partial [Coffea arabica L.]. 158aa	$9e^{-61}$	158/158 (100%)	158/158 (100%)
	CaC21	AHA93909.1 ethylene response factor 4, partial [Coffea arabica L.]. 165aa	$1e^{-109}$	165/165 (100%)	165/165 (100%)
	CaC26	AHA93910.1 ethylene response factor 11, partial [Coffea arabica L.]. 282aa	$6e^{-108}$	282/282(100%)	282/282(100%)
	CaC27	AHA93911.1 ethylene response factor 12 [Coffea arabica L.]. 252aa	$9e^{-147}$	252/252(100%)	252/252(100%)
	CaS36	AHA93913.1 ethylene response factor 15 [Coffea arabica L.]. 158aa	$1e^{-91}$	158/158 (100%)	158/158 (100%)

Table 2. Comparison of the EST-contigs and singlets with their best results obtained from BLASTx analysis in the NCBI database

**RT-qPCR Analysis of Relative Expression** The expression profiles of all the EST-*contigs* evaluated are shown in Fig. 5, with the relative expression levels of the sequences in the tissues of embryogenic callus, non-embryogenic callus, and embryogenic cells in suspension. The efficiency values for the *primers* used are shown in Table 1, with the reference genes used being the *ribosomal protein L39 (RPL39)* and the *ribosomal protein 24S (24S)* described by Freitas *et al.* (2017).

The expression profile of EST-*contig6* was highly expressed in embryogenic tissues (Fig. 5*a*). The expression level of the embryogenic cell suspension sequence was approximately 5.6 times higher than in embryogenic callus cultures, reaching 423 times when compared with non-embryogenic callus lines. The expression value for embryogenic callus cultures was 75 times higher than that were non-embryogenic.

The expression profile of EST-*contig9* (Fig. 5b) was similar to that of EST-*contig6*, where the highest expression was observed in embryogenic tissues. However, the level of EST-*contig9* expression in these tissues was lower than that observed in EST-*contig6*. The expression of the sequence in embryogenic callus cultures and embryogenic cell suspensions was 93 and 164 times higher, respectively, than non-embryogenic callus tissues.

Expression levels of EST-*contig27* were higher in the tissues of embryogenic callus cultures and embryogenic cell suspensions (Fig. 5*c*), where expression was 34 and 38.5 times higher, respectively, when compared with non-embryogenic callus cultures; however, there was no significant difference between embryogenic calluses and embryogenic cell suspensions.

## Discussion

**Analysis** *In Silico* The ERF/DREB family is characterized by having a unique AP2 domain (Nakano *et al.* 2006), whose size

is between 50 and 70 amino acid residues (Allen *et al.* 1998). The search for motifs of clustering for the sequences obtained in the CAFEST and Phytozome databases returned a motif whose size was 58 amino acid residues and was highly conserved among sequences (Figs. 6 and 7). The obtained motif was submitted to conserved domain analysis, confirming that it is the conserved region of the AP2 domain.

According to the amino acid sequence, the ERF family can be divided into two subfamilies corresponding to ERF (Nakano et al. 2006), with the classification based on the presence of two amino acids conserved in the AP2 domain. In Arabidopsis thaliana and cotton, the ERF subfamily was observed to have alanine (A14) and aspartic acid (D19) in the 14th and 19th positions, respectively, whereas the DREB subfamily was observed to have valine (V14) and glutamic acid (E19) in these same positions (Sakuma et al. 2002; Liu and Zhang 2017). Thus, the analysis of conserved amino acid residues of EST-contigs and singlets belonging to the ERF subfamily resulted in 15 EST-contigs and 5 singlets related to the subfamily ERF (Fig. 1a), in addition to 8 EST-contigs and 8 singlets relative to the DREB subfamily (Fig. 1b), corroborating the results obtained for the other previously mentioned species. For the Phy-contigs and Phy-singlets, the analysis allowed the selection of 54 (35 Phy-contigs and 19 Physinglets) and 50 (43 Phy-contigs and 7 Phy-singlets) sequences belonging to the ERF and DREB subfamilies, respectively (Fig. 2a, b). These results are important since they corroborate previous findings in the literature, contributing to an assertive division between the two subfamilies.

**Phylogenetic Analysis** Studies by Lima *et al.* (2011) on the genes related to the ethylene signaling pathway in coffee allowed the *in silico* identification of 13 *ERF* genes. Figure ure 3 shows that 12 EST-*contigs* and 1 *singlet* 





S34 (CA00-XX-FR1-074-C01-AG.F) S36 (CA00-XX-IA2-016-D09-EC.F) Figure 4. Electronic Northern representing the degree of expression of EST-contigs and singlets belonging to the ERF subfamily in the different libraries of Coffea arabica L. shown in grayscale where the darker the shade, the higher the degree of expression. The represented libraries are defined by Vieira et al. (2006). Libraries: BP1, cell suspension treated with acibenzolar-S-methyl; CB1, cell suspension treated with acibenzolar-S-methyl and brassinoesteroids; CL2, hypocotyls treated with acibenzolar-S-methyl; CS1, cell suspension treated with NaCl; EA1/IA/IA2, embryogenic calli; EM1/SI3, seeds at the beginning of germination; FB1/FB2/FB4, floral buds at different stages of flowering; FR1/FR2, flower buds and fruits in different stages; FR4, fruits (Coffea

racemosa Lour.); CA1/IC1/PC1, non-embryogenic calli; LV4/LV5, young leaves of orthotropic branches; RT8, cells in suspension with aluminum stress; SH2, field plants with stress from water deficit (tissue pool). The figure was generated by the program TreeView.

identified in the CAFEST database and the 9 Phy-contigs and 4 Phy-singlets identified in the Phytozome database are the same genes as those described by Lima et al. (2011), since they grouped together and were shown to be 100% identical (Table 2), being named according to the nomenclature proposed by Lima et al. (2011). This result was expected since the search performed in the Phytozome database used the PFAM number from the AP2 superfamily, allowing the identification of all sequences from this superfamily in this database. Similarly, in the CAFEST database, the same sequences identified by Lima et al. (2011) were also identified in this study, and this finding was expected, since the search on this database was performed using the sequence from the SERF1 gene, a member of the ERF subfamily (Mantiri et al. 2008b) that shares highly conserved domains characteristic of the ERF subfamily (Allen et al. 1998).

The unexpected result was the non-identification of the SERF1 gene in Coffea arabica in none of the databases analyzed because, as already mentioned, such a gene was the basis of the search in the CAFEST database. On the other



hand, in the Phytozome database, Phy-contig97 grouped with SERF1 from Medicago trucatula and Glycine max, indicating that this contig could represent the SERF1 from Coffea arabica. However, the global aligment through the ClustalW program (Thompson et al. 1994) of these sequences (Online Resource 1) and the comparison of the identity levels of Phy-contig97 with sequences from the NCBI database did not allow the assertive identification of this gene as the SERF1 from Coffea arabica. However, the possibility of the nonexistence of this gene in coffee should be considered since the SERF1 gene has been identified only in the species Medicago truncatula (Mantiri et al. 2008b) and soybean (Zheng et al. 2013).

Thus, despite the non-identification of the SERF1 gene, the discovery of 13 other genes in the CAFEST database belonging to the ERF subfamily was fundamental, since it allowed the analysis of possible involvement of these sequences in somatic embryogenesis in coffee. In addition, the identification of 108 sequences of the ERF subfamily in the Phytozome database suggests that other genes (not analyzed in our study) from this subfamily may possibly be involved in coffee somatic embryogenesis.

Expression Analysis by Electronic Northern The in silico expression profiles of ERF genes obtained in CAFEST corroborate those described by Lima et al. (2011); however, this author's work aimed at identifying genes that are members of the ethylene signaling pathway, and did not evaluate which of these genes may be related to the process of somatic embryogenesis. Thus, the EST-contigs and singlets that were expressed in the greatest number of embryogenic tissues, and in which they had their highest levels (EST-contig6, 9 and 27), were selected and evaluated in more detail.

Several studies have reported the expression of ERF genes in different tissues and treatments, and that they are responsible for several responses to biotic and abiotic stresses (Bouaziz et al. 2015; Dossa et al. 2016; Du et al. 2016; Liu and Zhang 2017); however, there have been few studies that have evaluated the involvement of these genes in somatic embryogenesis, with the studies by Mantiri et al. (2008a, 2008b), Zheng et al. (2013), and Silva et al. (2015) being the few available in the literature. Thus, the comparison of the selected EST-contigs and singlets with the genes described in these works can allow an evaluation of the importance of these sequences in the embryogenic process.

The expression of EST-contig6 in the libraries of the embryogenic calli (EA1/IA1/IA2) and cell suspension treated with acibenzolar-S-methyl and brassinosteroids (CB1) is interesting because Silva et al. (2015), when evaluating genes involved in somatic embryogenesis, identified a gene referred to as CaERF-like, which had a similar in silico expression profile. Likewise, the SERF1 gene, which belongs to the same subfamily, was identified in the study of Mantiri et al.

**Figure 5.** Profile of relative quantitative expression by qPCR of EST-*contigs* identified in *Coffea arabica* L. *Columns* represent the expression of gene transcripts in different tissues of coffee plants. (*a*) Quantitative expression of EST-*contig6*. (*b*) Quantitative expression of EST*contig9*. (*c*) Quantitative expression of EST-*contig27*. All gene transcripts were normalized by the expression of multiple reference genes (*RPL39* and 24S).



(2008b), in which the genotypes Jemalong 2HA and wild-type Jemalong of Medicago truncatula were molecularly analyzed, the first being a mutant 500 times more embryogenic than the second (Rose et al. 1999). In addition, the genes of the ERF subfamily are important in response to biotic and abiotic stresses. Thus, exposure to jasmonic acid, salicylic acid, ethylene, and abscisic acid implies the regulation of various plant defense-signaling pathways (Bouaziz et al. 2015), and may induce the process of somatic embryogenesis. The expression of EST-contig6 in the library of the cell suspension treated with acibenzolar-S-methyl and brassinosteroids (CB1) corroborates data from the literature, since acibenzolar-S-methyl is a pesticide that causes plant stress and brassinosteroid is a plant hormone, forming the combination of stress and an important hormone for early embryogenesis (Rose and Nolan 2006).

Like EST-contig6, EST-contig9 was expressed in the previously mentioned tissues. However, its expression in hypocotyls treated with acibenzolar-S-methyl (CL2) is relevant because the hypocotyl is a part of the stem of the embryo or seedling and the *SERF1* gene plays an important role in the induction of embryo formation (Mantiri *et al.* 2008a; Mantiri *et al.* 2008b), besides the ERF subfamily plays an important role in plant growth and development. In addition, hypocotyls were treated with acibenzolar-S-methyl, which is an exogenous substance and causes a defense response in the plant, such as the production of hormones. Kępczyńska *et al.* (2009) found that the addition of exogenous substances, such as abscisic acid, methyl jasmonate, and ethylene to culture media, improved the quality of *Medicago sativa* embryos. In the same work, these substances were related to the synthesis of ethylene, which is a hormone related to the expression of genes of the ERF subfamily.

In addition to the germinating seed library (whole seeds and zygotic embryos; EM1, SI3), EST-*contig27* was expressed in cell suspension treated with acibenzolar-Smethyl and brassinosteroids (CB1). This result corroborates Mantiri *et al.* (2008b), who analyzed the expression of the *SERF1* gene in somatic and zygotic embryos and observed that their expression in zygotic embryos was similar to the pattern observed in somatic embryos.

Electronic Northern analysis allowed the identification of three sequences, belonging to the ERF subfamily, ESTcontig6, EST-contig9, and EST-contig27, which may be



Contig2	:	FRGVRQRPWGKFAAEIRDPSKNGARVWLGTYETAEEAALAYDRAAYRMRGSKALLNFP : 5	58
Contig4	:	NYRGVRQRPWGKWAAEIRDPRKAARVWLGTFETAEDAARAYDRAAIEFRGPRAKLNFP : 5	58
Contig5	:	NYRGVRQRTWGKWVAEIREPKKGSRLWLGTFGTAIEAALAYDKAARVMYGPSAQLNLP : 5	58
Contig6	:	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTENTAEEAARAYDKEARKIRGKKAKVNFP : 5	58
Contig7	:	KYRGIRQRPWGKWAAEIRDPQKGVRVWLGTENTAEEAARAYDKAAKRIRGDKAKLNFV : 5	58
Contig8	:	NYRGVRQRTWGKWVAEIREPNRGSRLWLGTFGTAVEAALAYDEAARSMYGPCARLNLP : 5	58
Contig9	:	YRGVRRPWGKYAAEIRDSTRNGTRVWLGTFQTAEEAALAYDRAAFRMRGAKALLNFP : 5	58
Contig10	:	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDTEARRIRGKKAKVNFP : 5	58
Contig11	:	RGIRRRRWNSNKWVCELREPNKQSRIWLGTYPTAEMAARAYDVAALALRGHLACLNFA : 5	58
Contig12	:	HYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEAAARAYDTEARRIRGKKAKLNFP : 5	58
Contig15	:	HFRGVRKRPWGRYAAEIRDPSKKSRVWLGTFDTAEEAARAYDAAAREFRGPKAKTNFP : 5	58
Contig16	:	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAFKLRGDFTRLNFP : 5	58
Contig17	:	HFRGVRKRPWGRYAAEIRDPGKKSRVWLGTFDTAEEAARAYDAAARDFRGPKAKTNFP : 5	58
Contig18	:	VYRGVRMRAWGKWVSEIREPKKKSRIWLGTFACPEMAARAHDVAALSIKGNSAILNFP : 5	58
Contig20	:	RFRGVRKRPWGRFAAEIRDPWKKTRVWLGTFDSAEDAARAYDAAARTLRGPKAKTNFP : 5	58
Contig21	:	HFRGVRKRPWGRYAAEIRDPWKKTRRWLGTFDTAEEAALAYDEAARSLRGPKAKTNFG : 5	58
Contig22	:	KYRGIRQRPWGKWAAEIRDPQKGVRVWLGTFNTAEEAARAYDKAAKRIRGDKAKLNFV : 5	58
Contig23	:	LYKGIRMRKWGKWVAEIREPNKRSRIWLGSYSTPVAAARAYDTAVYYLRGPTARLNFP : 5	58
Contig24	:	TYRGVRQRTWGKWVAEIREPNHGARVWLGTFNTSYEAARAYDDAAKRLYGKCAKLNLP : 5	58
Contig26	:	RYRGVRQRPWGKWAAEIRDPHKAARVWLGTFDTAEAAARAYDEAALRFRGNRAKLNFP : 5	58
Contig27	:	YRGVRQRPWGKFAAEIRDPNRKGS <mark>RVWLGTF</mark> DTAVEAA <mark>KAYD</mark> KAAFRLRGSKAILNFP : 5	58
Contig28	:	HYRGVRQRPWGKWAAEIRDPKKAARVWLGTEETAEAAALAYDEAALRFKGNKAKLNFP : 5	58
Contig30	:	HFRGVRSR-SGKWVSEIREPRKTTRIWLGTYPTPEMAAAAYDAATLALKGPDAILNFP : 5	57
Singlet30	:	HYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEGAARAYDTEARRIRGKKAKLNFP : 5	58
Singlet36	:	HYRGVRQRPWGKWAAEIRDPKKAARVWLGTFETAEAAALAYDEAALRFKGNKAKLNFP : 5	58
Singlet34	:	HYRGIRQRPWGKWAAEIRRPRKGVRVWLGTFNTGEAAGRAYDTEARRIRRKKGNLNFP : 5	58
Singlet2	:	RYRGVRQRHWGSWVSEIRHPILKTRIWLGTFETAEDAARAYDEAARLMCGPRARSNFP : 5	58
Singlet3	:	AYKGVRQRTWGKWVAEIREPNRGSRLWLGTFDTSHEAAIAYDAAARKLYGTAAKLNLP : 5	58
Singlet6	:	KFRGVRQRHWGSWVSEIRHPLLKRRVWLGTFDTAEEAARAYDQAAVLMSGRNAKTNFP : 5	58
Singlet8	:	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEGAALAYDKAAFKLRSDFTRAELP : 5	58
Singlet10	:	KFRGVRQRPWGRWAAEIRDPTWKKRVWLGTFDTPEEAASAYDRAAVKLKGPDAVTNFP : 5	58
Singlet11	:	TYHGVRRRSWGKWVSEIREPRKKSRIWLGTFATPEMAARAHDVAAIAIKGHSAFLNFP : 5	58
Singlet21	:	FRGVRQRPWGKFAAEIRDPAKNGARVWLGTYETAXXAALAYDRAAYXMRGXKALLNSL : 5	28
Singlet31	:	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYTLRGEFARLNFP : 5	с 8 с
Singlet35	:	KFVGVRQRPSGKWVAEIKNTTHKIRMWLGTFDTAEETAQAYDEAACLLRGSNTRTNFM : 5	58
Singlet37	:	LYRGVRMRNWGKWVSETREPRKKSRTWLGTEPSPEMAARAHDVAALSTKGTSATINEP : 5	58

Figure 6. Grouping motifs of putative *Coffea arabica* L. genes belonging to the ERF subfamily found in the CAFEST database. The identity levels among the amino acid residues is represented by *grayscale*, where the *black color* indicates greater identity and the white color no identity levels.

related to the embryogenic process in *Coffea arabica* (Fig. 4). The sequences obtained were submitted to gene expression analysis by RT-qPCR (Fig. 5) in the tissues of embryogenic calli, non-embryogenic calli, and cell suspension. This analysis aimed to verify the relative expression levels of the three sequences in the mentioned tissues in order to gauge and suggest their influence on somatic embryogenesis.

**RT-qPCR Analysis of Relative Expression** The relative expression profiles obtained for the genes *CaERF8*, *CaERF13*, and *CaEFR12* were compared with that of the genes *MtSERF1* (Mantiri *et al.* 2008b), *GmSERF1*, *GmSERF2* (Zheng *et al.* 2013), and *CaERF-like* (Silva *et al.* 2015) due to the fact that these genes are important in somatic embryogenesis and belong to the same subfamily. This comparison aimed to



indicate the relationship of the identified genes with the embryogenic process, and to suggest possible molecular markers of this process in coffee.

The expression profiles of *CaERF8* and *CaERF13* are important because they attest to the results obtained in the Electronic Northern analysis, where the expression of these sequences in embryogenic tissues was observed. In addition, Mantiri *et al.* (2008b) evaluated the influence of ethylene on somatic embryogenesis and analyzed a genotype with high embryogenic capacity (Jemalong 2HA) and the wild-type (Jemalong) of *Medicago truncatula*. These authors identified the gene *MtSERF1*, the expression of which is induced by the presence of ethylene and is essential for the development of somatic embryogenesis. In the same study, the expression of the *MtSERF1* gene was verified in the two previously

Phy-Contig1	:	KYKGV	KRKWGKWVSEIRLPNSRERIN	GSYDSAEKAARAI	DAALFCIRCKNAKFNA : 58	Phy-Contig73	: YRGVRERPWGKFAAEIRDPKKKGSRIWLGTY	TPEEAAMAYDRAAFEIR	CARMMINEP : 58
Phy-Contig2	:	DOSRVI	ORSAGKWVAEIRDPHOGRSV	GTETSADEAARA	PRKCIOFREDRAKTNEP : 58	Phy-Contig76	RYRGVERRPWGRYAAEIRDPOSKERRWLGTR	TAPEAACAYDCAARAMR	CVKARTNEV : 58
Phy-Contig3		OSRRVI	ORSAGKWVAETRDPHOGRSV	GTETSAREAARA	TRACTOFREDRAKTNEP : 58	Phy-Contig77	HERGVEKRPWGRYAAETROPSKKSRVØLGTE	TAFEAARAYDAAAREFR	PKAKTNER : 58
Phy-Contig5	:	DEDCT	PREMERAUSETRURCTODEL	CSVAADEAAAMA	UPTEVYCEPENACIDEIAN · 59	Phy-Contig79	INDOVEDRING KNULLET DI DONDTEL MI CTEL	TAPPAALAVPPEAFEEP	FINDDINISD . 59
Dhu Contigo	:	KENGLA	OD DCCKWIZA ETKNITTOK TOXY	CUTDEARDAA	A REAL OF CHERRY AND THE STORE	Dhu Contig70	DYDCV ODUNCCHUCETDUDII KODINI CODI	TAR DAADA VOPADING	SDDDDCARD . 50
Phy-Contigo	•	VDCUD	DEVCKYA A DEDOCTORY	CULDIANEARO	POLYCHINGSNIKINHI . 50	Phy-Concig/9	I VIGTO VOCINUS DI DEDEDUVO CO VINCINI	THE DATE NOT DESCRIPTION	SPREATE . JO
Phy-Contigs	:	IRGVR	RPWGKYAAEIRDSTRKGIRVWI	GTEDSADAAALA	ADQMAFCMRCPMAVLNEP : 58	Phy-Contig82	: LIKGIRMRKWGKWVAEIREPNKRSRIWLGSI	TPVAAARAYDTAVYYDR	GPTØRLNFP : 58
Phy-Contig13	:	YRGVR	RPWGKYAAEIRDMNNNGAR	GTFAIIADEAARA	MDRAMFATRCSLMVLNDP : 58	Phy-Contig84	: YRGVRERPWGKYAAEIRDSTRNGTRVWLGTB	TADEAALAYDRAAFRMR	CAKALLNEP : 58
Phy-Contig15	:	KYRGV	QRPWGKWAAEIRDPARKARIW	GTYDHADEAAMV	YDRAAIQIRGPDALTNHI : 58	Phy-Contig85	: YRGVRERPWGKYAAEIRDSSRKGAR WLGTP	TADEAAMAYDKAALKIIR	CPKMYLNEP : 58
Phy-Contig16	:	KFRGVE	QRCWGSWVAEIRDPIKQKQVWI	GTYDIAEKAAIA	YDRAAIQFRGPKAITNEL : 58	Phy-Contig86	: NYRGVRQRTWGKWVAEIREPKKGSRLWLGTF	TAIEAALAYDKAARVMY	CPSAQLNLP : 58
Phy-Contig19	:	PYRGI	MRKWGKWVAEIREPNKRSRINI	GSYSSPVAAARA	YDTAVFYLRGPSARLNFP : 58	Phy-Contig87	: RYRGVERRPWGKWAAEIRDPYKAARVWLGTE	TADAAARAYDEAALRFR	CNKAKLNEP : 58
Phy-Contig20	:	AYKGV	ORTWGKWVAEIREPNRGSRUN	GTEDISHEAAIA	DAAARKIYCTAAKLALP : 58	Phy-Contig88	: PMYRGIRSRSGKWVSEIREPRKTTRUWLGTY	TPPMAAAAYDVAAIAIK	CSDIALNEP : 58
Phy-Contig21		TYKGV	ORTWGKWVAETREPNCGSRUM	GTEDTSHEAA TA	TAAAYKUYCEAAKINIP : 58	Phy-Contig90	KYTGVEKRKWRKYVSEIRLPNSRER WLGSY	TAPKAARAFDAALFCLR	KSWKENPP : 58
Phy-Contig22		YTGVR	RPWGKYAAETRDSTRNGMEV	GTEESPEEAALAT	YDOAAFSMRCPLASINEP : 58	Phy-Contig92	NYRGVEORPWGKWAAEIRDPRKAARVWLGDE	TAPDAARAYDRAAIEFR	CPRAKLNEP : 58
Phy-Contig22	:	VUCUDE	PDWCKEAAETPDCTPNCT	CERDEAREAATAN	POAL CMPODUAL MAD . 50	Phy=Contig93	HEDCVERDWCDVAAETDDDWKKTEDDUGTD	BAREANIAVERANDSER	PRAKTNING 58
Dhy-Contig24	:	VTCUDE	REPAIR AND TRACT PARTY OF THE PROPERTY OF THE PROPERTY AND THE PROPERTY OF THE	CTEDREROAALA	VEODARCHROTINCINER . 50	Phy-Contig90	UNDCT PDDNCCKWUCEUDEDNKKCPUNI CTE	TARMAADAHDVAATALD	EPCHCIMINA · 59
Phy-Contig24	•	VICUDA	RFWGRTAAEIRDSTRNGIR	GTEDREDQAALS	Constant Constant Constant	Phy Contigos	DUEDCUDCDCCKNUCEIDEDDKWWDIII CWU	Distance in the second second	CODDUTINATI - 50
Phy-Contig25	:	ILGVR	RPWGKIAAEIRDSTRNGLRVM	GTEDSABEAADA	DOWALAWREPATALWEP : 58	Phy-Concig95	PHERGVRSRSGRWVSEIREPRRIIK WEGTI	T POMAAAA TUAAT LATK	TEPDOTINE : 50
Phy-Contig28	:	YRGVR	RKSSGKWVSEIREPRTPNRLW	GIFPIPPMAAAA	IDVAALALKGPEAELNEP : 58	Phy-Contig96	KFRGV ORPWGKWAAEIRDPSTRAKIWLGTT	HADEAAMVYDNAAIKUR	CPDOLINEI : 58
Phy-Contig29	:	LYRGV	MRNWGKWVSEIREPRKKSRIW	GTFPSPEMAARA	HDVAALSIKGTSAILNEP : 58	Phy-Contig9/	: IRGVRERPWGKYAAEIRDPVRKGSRVWLGTFI	IIAMDAAKA IDSAAFKMR	GREATINEP : 58
Phy-Contig30	:	YRGVR	RPWGKYAAEIRDSTRNGVRVW	GTEDHADEAALV	YDQAAFALRGHAAVLNFP : 58	Phy-Contig98	YRGVRQRPWGKFAAEIRDPNRKGSRV MLGM	HAVEAAKAYDKAAFRI R	GSKAILNEP : 58
Phy-Contig31	:	NYRGV	QRTWGKWVAEIREPNRGSRIW	GTFGTAVEAALA	YDEAARSMYCPCARLNLP : 58	Phy-Contig99	FRGVRQRPWGKFAAEIRDPAKNGARVMLGTY	TADEAALAYDRAAYRMR	CSKALLNEP : 58
Phy-Contig32	:	LYRGV	ORHWGKWVAEIRLPKNRTRIM	GTEDTABEAALA	YDKAAYKLRGEFARLNEP : 58	Phy-Contig100	RYRGVRORSWGKWVAEIREPRKRTRRWLGTF	HADDAARAYDRAAIIIY	CSROQLNLQ : 58
Phy-Contig33	:	TYRGV	KRNWGKWVSEIREPRKKSRI	GTYPTADMAARA	HDVAALAIKCHSAYLNEP : 58	Phy-Contig101	RFRGVRKRPWGRFAAEIRDPWKKTRVWLGTFI	SAPDAARAYDAAARTLR	GPKAKTNEP : 58
Phy-Contig34	:	YRGVR	RPWGKFAAEIRNPNKKGSRU	GTYEEPEDAAMA	DLAAFEIRCARALLNEP : 58	Phy-Contig103	IYRGVEORHWGKWVAEIRLPRNRKRLWLGTF	TAPEAALAYDREAFRUR	GENARLNFP : 58
Phy-Contig35		YRGVR	RPWGTYAAETRDPKKKGSRI	GTYETPEDAALA	DOAAFKMRCARARVNER 58	Phy-Singlet3	YRGIR RPWGKYAAEIRDPTRNGAR WEGUN	TAPEAARAYDRAAYALR	CHOMLLNEP : 58
Phy-Contig37		KYRGT	ORPWGKWAAETRDPOKGVEV	GTENTAREAARA	YDKAAKRTRODKAKLNEV 58	Phy-Singlet4	LYRGIBORPWGKWAAEIRDPRKGVRVWLGTE	TAPEAARAYDKEARKIR	KKAKVNAP : 58
Phy-Contig38		RELOV	REPWORYAAFTEDETTKEEH	CTEDUAGEAALA	VERAALSMKCTOARTNEV 58	Phy-Singlet5	HYRGVEORPWGKWAAEIRDPKKPARVWLGDE	TAPAAALAYDEAALRFE	CNKAKLNEP : 58
Phy-Contig40	:	YRGVR	PPWGKYAAFIPDPTROGADU	CTERTAREAARA	KNAFNI BOHLATINER 58	Phy-Singlet6	KERGVISOR PWGRWAAETRDPTKRKRVWLGTY	TPEEAASVYDBAAVKLK	CPDAVTNEP : 58
Phy=Contig41		VDCUD	PPWGKFAAFIPDSTPNGIPWO	CTTD SAIDA AT AL	VPONAESMRGOANTINIAR . 58	Phy-Singlet8	YTGVRKRPWGKYAAETRDSTRNGMRVWLGDP	TAREAALAYDOAALSVR	CPTTALNER 58
Phy-Contig41	:	TEDCIN	OPUNCERNUS FIEL PONDTOWN	CERED BARRANDA		Phy-Singlet7	UNDOWERD DOCKNUSET DE DOKKTET NU OSEL	TERMANUAREAAAVEER	CAMOLNIAR · 58
Phy-Contig42		LINDCUS	ORHWGKWVAE IREPRINTIKUW	GITU ABLAAFA		Phy-Singlet9	VICUPADDWCKVAAEIDDSTDUCI PANIL CON	S DEFENSION VERSION	DMACINIAD 50
Phy-Contig43		VIRGVE	CIRSWGRWVSEIREPRRASE W	GIIS AJMAARA	HEVMALSHACKSHILLERPP : 56	Phy Cinglets	ONDETROPPICERIA A PED DDDKCUDWOU COD		GETTIGS HETE . 50
Phy-Contig44	•	KERGVE	QRHWGSWVSEIRHPLLKRRVW	GIFELABEAARA	NDEWSVIJUSJERNEKIMEP : 58	Phy-Singletiz	ONDELLOPPHERMAAEIRDPRKGVKVMLGTH	TABEAARS DIEDRAR	CREAK WEITE . 50
Phy-Contig45	:	NYRGV	KRPWGRWSAEIRDRIGRCRHW	GTEDHADEAARA	IDAAARKLIRGSKARTNIQ : 58	Phy-Singlet1/	QIRGINQRPWGRWAAEIRDPRRGVRVWLGIR	TABEAARA TO TEARRIR	CRACKWARP : 58
Phy-Contig46	:	RYRGV	QRHWGSWVSEIRHPLLKTRI	GTFEILADDAARAN	DEMARLACEPRERTNEA : 58	Phy-Singlet18	KFRGV©QRPWGKWAAEIRDPSRKVRIWLGTI	HABEAAMVNDHWD1QIIN	CPDELINEA: 58
Phy-Contig47	:	HYRGV	QRPWGKWAAEIRDPKKAARVW	GTFEHADAAALA	MDEAALRFKGNKAKLNEP : 58	Phy-Singlet19	RYLGVRRRQWGRYAAEIRNPYTKERHWLGTRI	MADEAAVAMDLASISFS	CIQKARTNF : 58
Phy-Contig48	:	YIGVR	RPWGKYAAEIRDSTRNGMRVWI	GTFATABEAALA	YDQAALSVRGPTTALNFP : 58	Phy-Singlet21	HYRGVERRPWGKWAAEIRDPKKAAEWWLGWG	MADDAALAYDEAALRFK	GTKAKINFP : 58
Phy-Contig49	:	RFVGV	QRPSGRWVAEIKDSSQRIRL	GTYDEPEEAARA	MDEAARALRGENARTNFA : 58	Phy-Singlet22	RFLGVERRPWGRYAAEIRDPTTKERHALGUN	II ADEAALAYDRAARSMR	GSRARINFV : 58
Phy-Contig50	:	HYRGI	QRPWGKWAAEIRDPRKGVRVWI	GTFNTAEGAARA	MDTEARRIRCKKAKLNFP : 58	Phy-Singlet23	RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFI	TADEAALAYDRAARSMR	CSRARTNEV : 58
Phy-Contig52	:	EYRGV	ORTWGKWVAEIREPKKRTRI	GSFATABEAAMA	DEAARRIYCPDAYVNLP : 58	Phy-Singlet24	KFRGVRQRQWGSWVSEIRHPLLKKRIWLGTF	TADAAARAYDQAAILMN	ICQNAKTNEP : 58
Phy-Contig54	:	LYRGV	QRHWGKWVAEIRLPQNRMRVWI	GTYDTAEAAAYA	YDRAAYKLRGEYARLNFP : 58	Phy-Singlet25	KFRGVRQRQWGSWVSEIRHPLLKKRIWLGTP	TADAAARAYDEAAILMN	GQNAKTNFP : 58
Phy-Contig55	:	YRGVR	RPWGKFAAEIRDPAKNGARVWI	GTFETAEDAAIA	MDRAAYRMRCARALLNEP : 58	Phy-Singlet26	RFVGVRQRPSGRWVAEIKDTIQKIRVWLGTF	TADEAARAYDEAACLLR	CANTRINEW : 58
Phy-Contig56	:	YRGVRC	RPWGKYAAEIRDPKRRGSRV 71	GTEDIAIEAAKA	MDRAAFKMRCSKAILNEP : 58	Phy-Singlet27	RYRGVRORPWGKWAAEIRDPHKAARVWLGTP	TADAAARAYDEAALRFR	CNRAKLNFP : 58
Phy-Contig57	:	TYHGVI	RRSWGKWVSETREPRKKSRU	GTRATPEMAARA	HIVAAIAIKCHSAFLNEP : 58	Phy-Singlet28	KFLGVERRPWGRFAAEIRDPTTKERHNEGDE	TAPEAALAYDKAARSMR	CARARTNAV : 58
Phy-Contig58	-	RGIRR	RWNSNKWVCELREPNKOSRT	GTYPTAPMAARA	YDVAALALRGHLACINFA : 58	Phy-Singlet29	KELGVERRPWGRFAAEIRDPTTKEEHWLGDE	TAPEAALAYDKAARSMR	CSRARTNEV : 58
Phy-Contig59		KMKGW	MRSWGSWVSETRAPNOKTET	CSYSTAPAAARA	AALLCIKCSSANIMER · 58	Phy-Singlet 30	RYRGVEORPWGKWAAETRDPHKAARVWLGTPI	TAPAAAAAAYDEAALRER	CNRAKINAP · 58
Phy-Contig60		KYVGV	OPPSCRWVAFIKDTTOKIEM	CTEFTARFAARA	VERACLIECSNTRTNEV 58	Phy-Singlet 33	RYKGVEMRKWGKWVAEVROPKSRDELWLGSY	DAREARRAYDAAAFCIR	PSVMLMAP · 58
Phy=Contig61	:	SYTCT	OPKWCKWVSETPEPCKKSPT	CSYFADELAAAA		Phy-Singlet 34	VEGTERPWGKYAAFTEDPTENGARI WIGTEI	DAREAARAYDRAAYALB	CHOMLINIAP · 58
Dhu-Contig62	:	INDOW	ORUMORWY SETREF ORROATIN	CERDEAREAATA	VERAFEL PODETDINED . 50	Phy-Singlet25	UNDOWNOR DECKINA A FIRDERY DA DUNIL CTEL	DADA ATAY DEAT DEE	CNIVER INTER . 59
Phy-Contig62	•	DIRGVE	ODTHORNWARDEDEDNUCA	CUDNECYPANDA	DEVELOPTION F : 58	phy-Ginglet20	DYKCUMPYNCKWUZEVDODKCDDDTWLCCX	TAREA DAY DAY DO	DOUMLAND . EQ
Phy-Contig63	1	UNDOW	WE WERWVAE I REPNHGARVWI	GILLINESILAAR	UNITERCOKLALP : 58	Phy-Singlet38	KENCK ONDERGENUARICOPKSKUKI WIGSH	A A A A A A A A A A A A A A A A A A A	CPSVPIEREP : 38
rny-Contig65	•	VIRGV	TRAWGRWVSEIREPRRKSRIWI	GIRACPEMAARA		rny-Singiet39	KINGARÓMKMCZMANTCDEAKŐKŐAMPCLA	HITEL MANAGEMENT REPR	CENCELINEE : 58
Phy-Contig66	•	NF KGV	QKHWGSWVSEIKHPLLKRRVV	GIRDIAREAARA	DOGTOVLOSCKNOKINTER : 58				
Pny-Contig68	:	RYVGV	QRPSGRWVAEIKDSLQKVRL	GTH DINAP DAARA	IDEAARALIKGANARTNEE : 58				
Phy-Contig69	:	RFLGV	QRPSGRWVAE1KDSSQKLR	GIRDNADEAALA	DNAARLIRGRNAKTNET : 58				
Phy-Contig70	:	HFRGV	KRPWGRYAAEIRDPGKKSRV	GIEDIADEAARA	DAAAREFRGAKAKTNEP : 58				
Phy-Contig71	:	HYRGV	KRPWGRYAAEIRDPWKKTRVW	GTFDIPEEAALA	IDGAARSLEGAKAKINFP : 58				
Phy-Contig72	:	HFRGVE	ORSEGKWVAEIRNPHLGRTE	GTFTSADEAARAI	RKCIOYKEDREKTNEP : 58				

Figure 7. Grouping motifs of putative Coffea arabica L. genes belonging to the ERF subfamily found in the Phytozome database. The identity levels among the amino acid residues is represented by grayscale, where the black color indicates greater identity and the white color no identity levels.

mentioned genotypes and a high expression was observed in the highly embryogenic genotype, an observation not noticed in the other genotype, thus confirming its importance in the induction of embryo formation.

The expression levels of CaEFR12 were found to be higher in embryogenic tissues. This result is interesting because, as already mentioned, the SERF1 gene is essential for the development of somatic embryogenesis (Mantiri et al. 2008b), and so a high expression of the sequence in this tissue was expected, which was indeed observed. In soybean (Glycine max), Zheng et al. (2013) identified orthologs of the SERF1 gene that act together with the gene AGAMOUS-like15 in the induction of somatic embryogensis. Analyzing the gene expression of the orthologs identified in soybean, this author obtained the greatest expression of this sequence in the tissue of explants of immature cotyledons cultivated in culture medium inducing somatic embryogenesis, thus confirming the importance of this gene in this process, besides corroborating the result obtained for the CaEFR12 gene.

The only studies of the SERF1 gene available in the literature (Mantiri et al. 2008a; Mantiri et al. 2008b; Zheng et al. 2013) report it as a gene that acts directly on the process of somatic embryogenesis, being highly expressed in embryogenic tissues and with low expression in non-embryogenic tissues. In addition, Mantiri et al. (2008a) suggests that MtSERF1 shows biding sites to the promoter region of the WUSCHEL (WUS) gene, possibly

acting in conjuction with this gene to regulate genes involved in somatic embryogenesis onset. The SERF1 gene from Coffea arabica was not identified in this study. Therefore, the comparison of its expression with the one observed for Coffea arabica WOX (WUSHEL-related HOMEOBOX) genes (Daude et al. 2020), which would show whether these genes act in a pattern similar to one observed by Mantiri et al. (2008a), could not be performed.

The expression profile obtained for the genes CaERF8, CaERF13, and CaEFR12 attest to the results described in the cited studies, indicating that these genes may be intrinsically linked to the mechanism of somatic embryogenesis in coffee. In addition, the expression of the genes CaERF8 and *CaERF13* exclusively in embryogenic tissues is important, as it suggests the potential use of these sequences as molecular markers of somatic embryogenesis. Studies on genomic analysis during somatic embryogenesis in coffee have suggested the identification and characterization of two molecular markers of the embryogenic process: the genes somatic embryogenesis receptor-like kinases (SERK1) and baby boomlike (BBM; Silva et al. 2014, 2015).

The function of the gene SERK1 is correlated with the activation of the embryogenic process in somatic or zygotic cells through the identification of signaling molecules, in addition to its expression from the earliest stages of embryonic development of the globular stage of the embryo (Schmidt



*et al.* 1997; Ikeda *et al.* 2006). Based on their function, homologs have been described in several species (Hecht *et al.* 2001; Nolan *et al.* 2003; Yang *et al.* 2011; Ma *et al.* 2012). In transcriptional analyses of *SERK1* in *Arabidopsis* and coffee, Hecht *et al.* (2001) and Silva *et al.* (2014) obtained high gene expression in embryogenic tissues, if not observed for non-embryogenic tissues, thus proposing its use as a marker of the embryogenic process. However, even though it is a gene with its highest expression in embryogenic tissues, the fact that it has been expressed in non-embryogenic tissues could be a hindrance to its application as a molecular marker, since this may generate false positives.

Comparing the expression profile obtained for the genes CaERF8 and CaERF13 with the gene SERK1, identified and characterized in coffee as a molecular marker of somatic embryogenesis, a great difference in expression profiles can be observed. Expression of the gene CaSERK1 described by Silva et al. (2014) in the suspension of embryogenic cell tissue is approximately 10-fold lower when compared with the gene CaERF8, and 6-fold lower for the gene CaERF13. A significant difference in the expression level of these genes in embryogenic calli tissue is also observed, with the genes CaERF8 and CaERF13 being 7 and 14 times more expressed than the *CaSERK1* gene, respectively. This comparison is important because it suggests that CaERF8 and CaERF13 are superior molecular markers than CaSERK1, since their expressions in embryogenic tissues were superior. This result is fundamental, as it can direct the study of these genes in other species in order to evaluate the embryogenic capacity of the species or material used.

In the work of Silva et al. (2015), the same evaluation of the expression of the gene CaBBM in tissues similar to that used in the present study obtained high expression in the embryogenic tissues, and no expression in non-embryogenic tissue. This expression profile resembles that obtained for the genes CaERF8 and CaERF13, demonstrating the possibility of using these as molecular markers since they belong to the same superfamily (AP2/EREBP). However, an important factor in the selection of a marker gene is the function the gene performs during the process, with BBM being related to cellular proliferation and embryogenic processes (Passarinho et al. 2008; El Ouakfaoui et al. 2010), while genes belonging to the ERF subfamily are related to plant growth and development, as well as responses to biotic and biotic stresses (Bouaziz et al. 2015). Thus, the analysis of these factors is important because, depending on the stage of tissue development, they can directly influence the use of these genes as molecular markers. The results obtained in the analysis of the relative expressions of the genes CaERF8 and CaERF13 will enable future studies aimed at validating these genes as molecular markers, as well as evaluating their relationship to the regulation of somatic embryogenesis Coffea arabica.

### Conclusion

The EST-contig6, 9, and 27 were found to correpond to *CaERF8*, *CaERF12*, and *CaERF13* genes from *Coffea* arabica. The RT-qPCR analyses of relative expression of the identified genes indicated that *CaERF8*, *CaERF12*, and *CaERF13* could be directly linked to somatic embryogenesis. In addition, the expression levels of *CaERF8* and *CaERF13* in tissues of embryogenic callus cultures and embryogenic cell suspensions indicate that they may be markers of somatic embryogenesis in Arabic coffee.

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