EXPRESSION ANALYSIS OF THE COFFE (*Coffea arabica L.*) *FRIGIDA4-like* GENE (*CaFRL4*)

Análise da expressão do gene FRIGIDA4-like (FRL4) de café (Coffea arabica L.)

Análisis de la expresión del gen FRIGIDA4-like (FRL4) de café (Coffea arábica L.)

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ABSTRACT

Coffee is one of the most economically important commodities. In Brazil, this crop is responsible for generating more than eight million jobs. In the foreign market, Brazil is the largest producer and exporter of coffee. Due to its economic importance, several studies aiming the improvement of coffee are conducted, but there are still problems related to its productivity and quality of the beverage, such as sequential flowering, which causes production losses and a low quality drink. Thus, understanding the molecular mechanisms involved in the flowering process is essential to elucidate how flowering occurs in the coffee crop. The FRI gene is one of the main genes involved in flowering, as it positively regulates the FLC gene at expression levels that inhibit flowering. Thus, the objective of this work was to identify and analyze the expression of the FRIGIDA4-like gene (FRL4) through Bioinformatics and real-time PCR (RT-qPCR). The CaFRL4 gene was identified and showed high expression levels in leaf during flowering, which corroborates with the literature. The results obtained provide the basis for future studies involving genetic transformation in model plants and coffee, permitting the functional characterization of this gene.

Keywords: RT-qPCR, Arabica coffee, Flowering.

RESUMO

O café é uma das commodities de maior importância econômica. Em âmbito nacional, a cafeicultura é responsável pela geração de mais de oito milhões de empregos. No mercado externo, o Brasil é o maior produtor e exportador do café. Diante da sua importância econômica, vários estudos visando o melhoramento do cafeeiro são desenvolvidos, mas, ainda existem problemas relacionados a sua produtividade e qualidade da bebida, como o florescimento sequencial, que ocasiona perdas de produção e uma bebida de baixa qualidade. Deste modo, a compreensão dos mecanismos moleculares envolvidos no processo de florescimento é essencial para elucidar como o florescimento ocorre na cultura do café. O gene FRI é um dos principais genes envolvidos no florescimento, pois regula positivamente o gene FLC a níveis de expressão que inibem o florescimento. Dessa forma, o objetivo deste trabalho foi identificar e analisar a expressão do gene FRIGIDA4-like (FRL4) por meio da Bioinformática e da PCR em tempo real (RT-qPCR). O gene CaFRL4 foi identificado e apresentou altos níveis de expressão em folha durante o florescimento, o que corrobora com a literatura. Os resultados obtidos dão base para estudos futuros envolvendo transformação genética em plantas-modelo e em café, possibilitando a caracterização funcional desse gene.

Palavras-chave: RT-qPCR; Café árabica; Florescimento.

RESUMEN

El café es uno de los commodities de mayor importancia económica. A nivel nacional, la cafeicultura es responsable de la generación de más de ocho millones de empleos. En el mercado externo, Brasil es el mayor productor y exportador del café. En cuanto a su importancia económica, varios estudios para mejorar el café se desarrollan, pero todavía existen problemas relacionados con su productividad y calidad de la bebida, como el florecimiento secuencial, que ocasiona pérdidas de producción y una bebida de baja calidad. De este modo, la comprensión de los mecanismos moleculares involucrados en el proceso de florecimiento es esencial para dilucidar cómo el florecimiento ocurre en el cultivo del café. El gen FRI es uno de los principales genes involucrados en el florecimiento, pues regula positivamente el gen FLC a niveles de expresión que inhiben el florecimiento. De esta forma, el objetivo de este trabajo fue identificar y analizar la expresión del gen FRIGIDA4-like (FRL4) a través de la Bioinformática y la PCR en tiempo real (RT-qPCR). El gen CaFRL4 fue identificado y presentó altos niveles de expresión en hoja durante el florecimiento, lo que corrobora con la literatura. Los resultados obtenidos dan base para estudios futuros involucrando transformación genética en plantas modelo y en café, posibilitando la caracterización funcional de ese gen.

Descriptores: *RT-qPCR, Café Arábigo, Floración.*

INTRODUCTION

Coffee is one of the commodities with most economic importance in the world. In 2016, approximately 158 million 60 kg bags were consumed worldwide, with European Union, USA, and Brazil posing as the main consumers (ICO, 2016). Displaying a planted area of 2.223.464,1 hectares a production of 51.37 million bags of 60 kg (CONAB, 2016), Brazil is the biggest coffee producer, followed by Colombia, Indonesia, and Vietnam (ICO, 2016). Coffee belongs to the *Rubiaceae* family and displays two species with economic importance: *Coffea arabica* and *Coffea canephora*, with the first being responsible for about 70% of coffee production in the world. However, though its high production, Brazilian coffee shows a lower price when compared to other countries (ICO, 2016).

The decrease in final price of coffee is due to productivity problems that, though extensively studied, it still poses as a problem to this crop. Coffee trees shows a sequential flowering, leading to the asynchronous fruit ripening, causing difficulties in the harvest, production losses, and a decrease in cup quality (CHALFOUN, 2010). Thus, flowering synchronization ripening uniformity can significantly contribute to the reduction in production costs and increase cup quality.

Coffee flowering initiation is a result of phenological patterns of the crop, as well as the interaction with environmental factors like water availability, photoperiod, and irradiation levels (WANG et al., 2011). Therefore, the comprehension identification of the mechanisms involved in the flowering process, as well as the quantification of the expression level of genes involved in the flowering regulation pathways, are of fundamental importance to elucidate how this developmental process takes place in coffee trees. Thus, the study of the genes responsible for the control of the flowering process is an important tool to the flowering and fruit ripening synchronization, being essential to better comprehend these genetic factors and how they act under the new climatic and geographical conditions (BLUEMEL et al., 2015).

The *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) genes were identified in the flower regulation pathway of vernalization in *Arabidopsis*, where *FRI* acts in the regulation of *FLC* in a positive way, repressing plant flowering (CHAO et al., 2013). The *FRIGIDA* protein possess spiral domains coiled at two positions of its structure, stablished between the

amino acids 55-100 and 405-450, respectively, and these *FRI* domains are necessary to increase the transcription of *FLC* (CHAO et al., 2013). The *FRI* gene acts as a recruiter protein that interacts with *FRIGIDA-like1* (*FRL1*), *FRIGIDA-like1* (*FRL1*), *FRIGIDA ESSENTIAL 1* (*FES1*), *SUF4*, and *FLC EXPRESSOR* (*FLX*), to form a complex (*FRI-C*), which activates transcription, recruiting chromatin modifiers to regulate *FLC* (CHOI et al., 2011; GERALDO et al., 2009).

Recently, it was stablished the *FRI* structure of *Vitis vinifera*, and this structure can serve as a structural basis for the understanding of floral regulation and also for the determination of *FRI* structure in other species (HYUN et al., 2016). The proteins of the *FRI* family show a conserved central region and can be classified into five subfamilies according to their N-terminal region. The C-terminal region is essential for *FRI* function and thought that this region mediates protein:protein interactions (RISK et al., 2010).

Most studies about *FRI* genes were carried out in Arabidopsis thaliana and these genes have been identified only in some species. Chao et al., (2013) identified a *FRIGIDA-like* gene, *MsFRI-L*, in *Medicago sativa* L. and showed that this gene can delay flowering time through the regulation of gene involved in flowering.

After the coffee transcriptome sequencing (VIEIRA et al., 2006) and the creation of a database of sequences that encodes for putative genes related to characteristics of interest, comparative studies of functional genomics emerged as powerful tools for the stud of genes involved in the regulation of metabolic pathways. However, studies related to gene expression in coffee trees are still scarce. Thus, this study aimed to analyze the expression pattern of the *FRIGIDA4-like* (*FRL4)* in arabica coffee, in order to better comprehend its function in coffee flowering regulation.

MATERIAL AND METHODS

In silico analysis

Putative coffee *FRIGIDA4-like* (*FRL4*) homolog gene was obtained from data mining in the coffee (*Coffea arabica*) expressed sequence tag (EST) database (CAFEST) (bioinfo04.ibi.unicamp.br), composed by 214,964 ESTs distributed into 37 cDNA libraries sequenced from the 5′ end (VIEIRA et al., 2006). Data mining in the CAFEST database was carried out using *FRL4* gene (BLASTn) and protein (tBLASTn) sequences as baits, as well as keyword searches. The sequences with significant similarity (*e-value* $<10^{-4}$) were selected and sent to the sequence manager and manipulation system, the GeneProject, and submitted to clustering using the CAP3 program (HUANG and MADAN, 1999), forming the EST-*contigs* and *singlets*. Data validation was performed by local tBLASTx and tBLASTn searches of the retrieved sequences against the GenBank database. The open reading frame (ORF) of the validated sequences was obtained through the ORFinder tool (NCBI).

Phylogenetic and *in silico* expression analyses

Protein sequence alignments were performed by the ClustalW program (THOMPSON et al., 1994) using default parameters, and phylogenetic trees were generated by the MEGA software, version 4.0 (TAMURA et al., 2007), with neighbor-joining comparison model (SAITOU and NEI, 1987), p-distance method and pair-wise suppression. Bootstrap values from 10,000 replicates were used to assess the robustness of the trees.

In silico qualitative gene expression profiling was performed using virtual *Northern* blot analyses of the coffee EST database. The frequency of *reads* from each EST-*contig* and *singlet* in the CAFEST libraries was calculated, and data normalization enabled the comparison of gene expression in each treatment and plant organ. Normalization consisted of multiplying

each *read* by the ratio between the total number of *reads* from all libraries and the *read* number of the library where it was expressed. The results were plotted in a matrix and gene expression patterns among ESTs and libraries were obtained by hierarchical clustering, performed by the Cluster v.2.11 program (EISEN et al., 1998). Graphic outputs were generated by the TreeView v.1.6 software (EISEN et al., 1998).

Conserved domain analysis

In order to discover the conserved domains among the putative coffee *FRL4* protein sequences encoded by the sequences found in the CAFEST database and from *FRL4* sequences from other species, the Conserved Domain program, from the NCBI database (ncbi.nlm.nih.gov/), was used. Complete amino acid sequences were compared according to the clustering order observed in the phylogenetic tree, enabling the assessment of whether all studied sequences belonged to the same gene superfamily.

Plant material

Plant material was harvest at the experimental field of the Federal University of Lavras, Lavras-MG (Brazil) (21°14'42"S 45°00'00"W), and the experimental analysis were carried out at the Laboratory of Molecular Analysis of the Federal University of Tocantins, Palmas-TO. RNA extraction was performed in four *Coffea arabica cv* Rubi tissues: roots (secondary roots), leaves (during plant flowering), flower buds (G4-G5 developmental stages (MORAIS et al., 2008), and fruits. Samples were harvested and immediately frozen in liquid nitrogen and futher stored at -80 °C.

RNA extraction and cDNA synthesis

Total RNA was extracted from the tissues using Invitrogen Concert™ Plant RNA reagent (Life Technologies, Carlsbad, CA, USA), and 5 μg of the resulting extracts was treated with Ambion® Turbo DNA- free kit reagents (Life Technologies) to remove any contaminating genomic DNA. The quantity and the purity of total RNA were assessed with the aid of a spectrophotometer (Nanodrop® ND-1000), while RNA integrity was visually analyzed in 1 % agarose gel. The cDNA was synthesized from 1.0 μg of DNA-free RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol.

Reverse Transcription-quantitative Real Time PCR (RT-qPCR)

RT-qPCR was performed using 10 ng of cDNA in a 10 μL reaction volume with SYBR Green UDG Master Mix with ROX (Invitrogen) on an ABI PRISM 7,500 real-time PCR thermal cycler (Applied Biosystems). Based on the sequence obtained in the *in silico* analysis, primers for the putative coffee *FRL4* gene (Table 1) were designed using the Primer Express v2.0 program (Applied Biosystems). RT-qPCR conditions were as follows: 95 °C (15 min), then 40 cycles of 95 and 60 °C (15 s), followed by 1 min at 60 °C, and completed with a melting curve analysis program. Three biological replicates for each tissue type were used, and reactions were run in triplicates. Relative fold differences were calculated based on the ΔΔCT method (PFAFFL, 2001), using *Ubiquitin* (*UBQ*) and the *Ribosomal protein L39* (*RPL39*) as reference genes (Table 1) (FERNANDES-BRUM et al. 2017), and were calculated relative to a calibrator sample, which was selected based on the lower expression level of the putative coffee *FRL4* among the tissues analyzed.

Table 1. RT-qPCR primer sequences, accession numbers (Genbank), and gene amplification efficiencies.

Gene	Accession number	Primer sequence (5' to 3')	Amplification efficiency (%)
CaFRL4	HQ845335.1	Fw: ATGGACACGGAAGCAGGA Rv: AGGATACGTTACTGGAGCTC	93
UBQ	KP770021	Fw: AATCCGTCCCCGCATGTT Rv: CCAGTGCATCCTGTTGTCTCA	99
RPL39	GT720707.1	Fw: GCGAAGAAGCAGAGGCAGAA Rv: TTGGCATTGTAGCGGATGGT	87

RESULTS AND DISCUSSION

In silico analysis

The search for *reads* in the CAFEST database yielded 57 *reads* that after clusterization formed 6 EST-*contigs* and 7 *singlets*. After the analysis of the conserved domains of their protein sequences, it was observed that two EST-*contigs* (1 and 5) and one *singlet* (CA00-XX-LV8-086-H01-CE.F) showed the *FRIGIDA* (*FRI*) domain (Figure 1b). The EST-*contigs* and *singlets* that did not show the *FRI* domain, which characterizes the *FRIGIDA* superfamily, were excluded of the further analysis.

Phylogenetic analysis

The phylogenetic analysis showed the comparison between the EST-*contigs* 1 and 5 and the *singlet*, with the genes that belongs to the *FRIGIDA* superfamaily characterized in other species and used in the study of Risk et al., (2010). In figure 1a, one can observe that the EST-*contigs* 1 and 5 and the *singlet* clustered on the same clade and showed similarity with *FRL4* genes from *Medicago truncatula, Glycine max, Vitis vinifera* and *Arabidopsis thaliana.*

The *FRIGIDA* superfamily can be divided into five families, which are classified based on their conserved amino acid sequences. However, despite this division, all genes from this superfamily possess the conserved *FRI* domain (RISK et al., 2010). In figure 1b, it is possible to observe that all sequences used in

the phylogenetic analysis have the *FRI* domain. This is important since it corroborates with the literature and proves that every sequence used in this analysis belong to the *FRI* superfamily.

In *Arabidopsis*, Risk et al., (2010) have identified an N-terminal conserved region that characterizes each of the five families that belong to the *FRIGIDA* superfamily. This region was also found in other species (RISK et al., 2010). Figure 2 represents the global alignment of the sequences used in the phylogenetic analysis, showing the similarity among them, as well as the conserved region that characterizes the *FRIGIDA4-like* (*FRL4*) family. Thus, the conserved region (red line) at the N-terminal portion of the protein could be observed in all sequences, except for the *singlet* (CA00-XX-LV8-086-H01-CE.F). This can be related to the assembling of the sequences, since this process can lead to the formation of partial sequences, considering that it generates a new sequence from a group of several *reads*.

In this way, figure 2 enabled the identification that the EST-*contig5* is a strong candidate of the coffee *FRL4* homolog gene, considering that it displayed elevated similarity with *FRL4* genes described in other species, it shows the N-terminal conserved region that characterizes the *FRL4* family, and it shows a protein size similar to other *FRL4* genes described in other species.

Figure 1. Phylogenetic analysis (a) and conserved domain analysis (b) of the *FRL4* genes from *Medicago truncatula*, *Glycine max*, *Vitis vinífera*, *Arabidopsis thaliana*, *Oriza sativa*, found in the NCBI database, and the putative coffee *FRI4* genes, the EST-*contig1*, the EST-*contig5*, and the *singlet* (CA00-XX-LV8-086-H01-CE.F), found in the CAFEST database. (●) EST-*contigs*, (○) *singlet*, (■) *FRL4* genes described in other species. Neighbor-joining comparison model, p-distance method and pair-wise suppression were used for the construction of the phylogenetic tree. Bootstrap values from 10,000 replicates were used to assess the robust- ness of the tree.
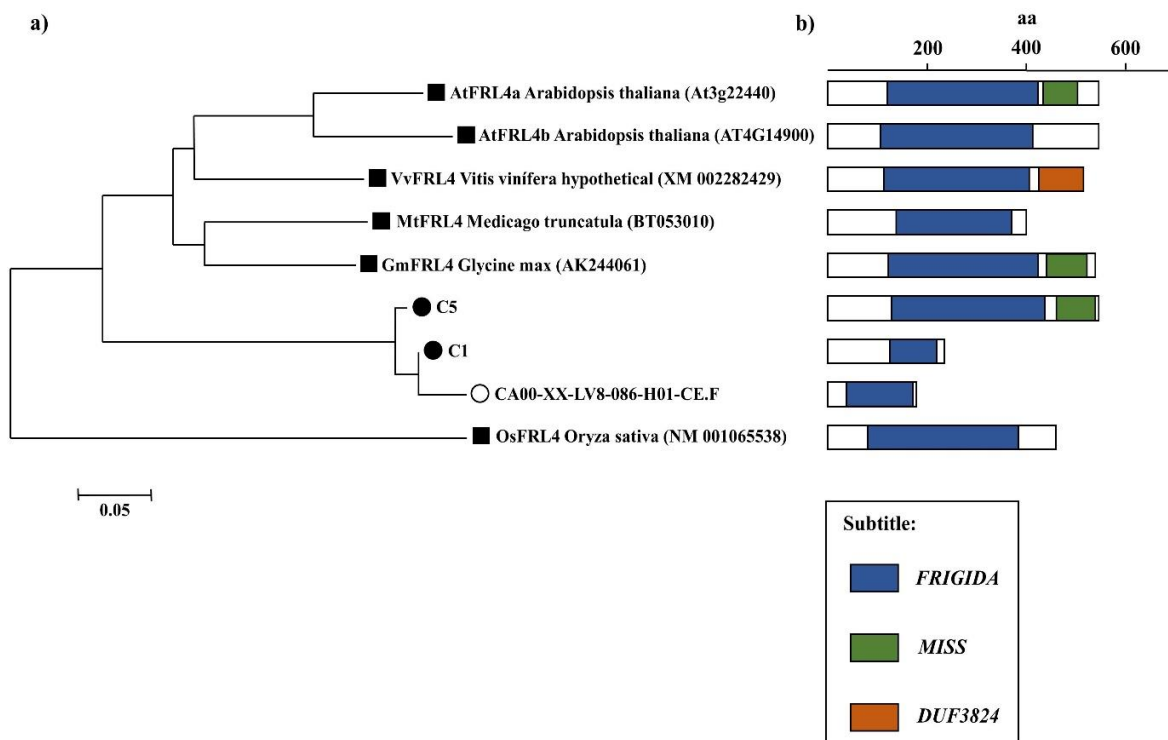

In silico gene expression

The electronic northern of the three sequences obtained in the CAFEST (EST-*contig1*, EST-*contig5*, and the *singlet*) is represented in figure 3. It can be observed that the EST-*contig1* was expressed in seed tissues at the beginning of germination (EM1/SI3), non-embriogenic calli (CA1/IC1/PC1), mature leaves from plagiotropic branches (LV8/LV9), and suspension cell under aluminum stress (RT8). EST-*contig5* showed the broader expression pattern, since its expression could be detected in all libraries in which the expression of the three coffee *FRL4* candidate genes was detected, except for mature leaves from plagiotropic branches. The *singlet* (CA00-XX-LV8-086-H01-CE.F) was expressed in leaf tissue (LV8/LV9).

The expression pattern observed for EST-*contig5* is important, since it corroborates with the literature, considering that it was expressed several tissues and, Risk et al., 2010 describes that family IV genes display high expression levels in many tissues, including developing seeds. Thus, based on the expression pattern found, EST-*contig5* was identified as the putative coffee *FRL4* homolog gene, being named as *CaFRI4-like* (*CaFRL4*).

Figure 2. Global alignment among the amino acid sequences encoded by *FRL4* genes described in other species (*AtFRL4a*, *AtFRL4b*, *MtFRL4*, *GmFRL4*, *VvFRL4* e *OsFRL4*) with the coffee EST-*contig1*, EST-*contig5*, and the *singlet* (CA00-XX-LV8-086-H01-CE.F). The red line represents the region that characterizes the *FRL4* family (RISK et al., 2010). Sequence alignment performed by the ClustalW program and the image was generated by the GeneDoc program. Identical and conserved amino acid are shown in black and grew, respectively.
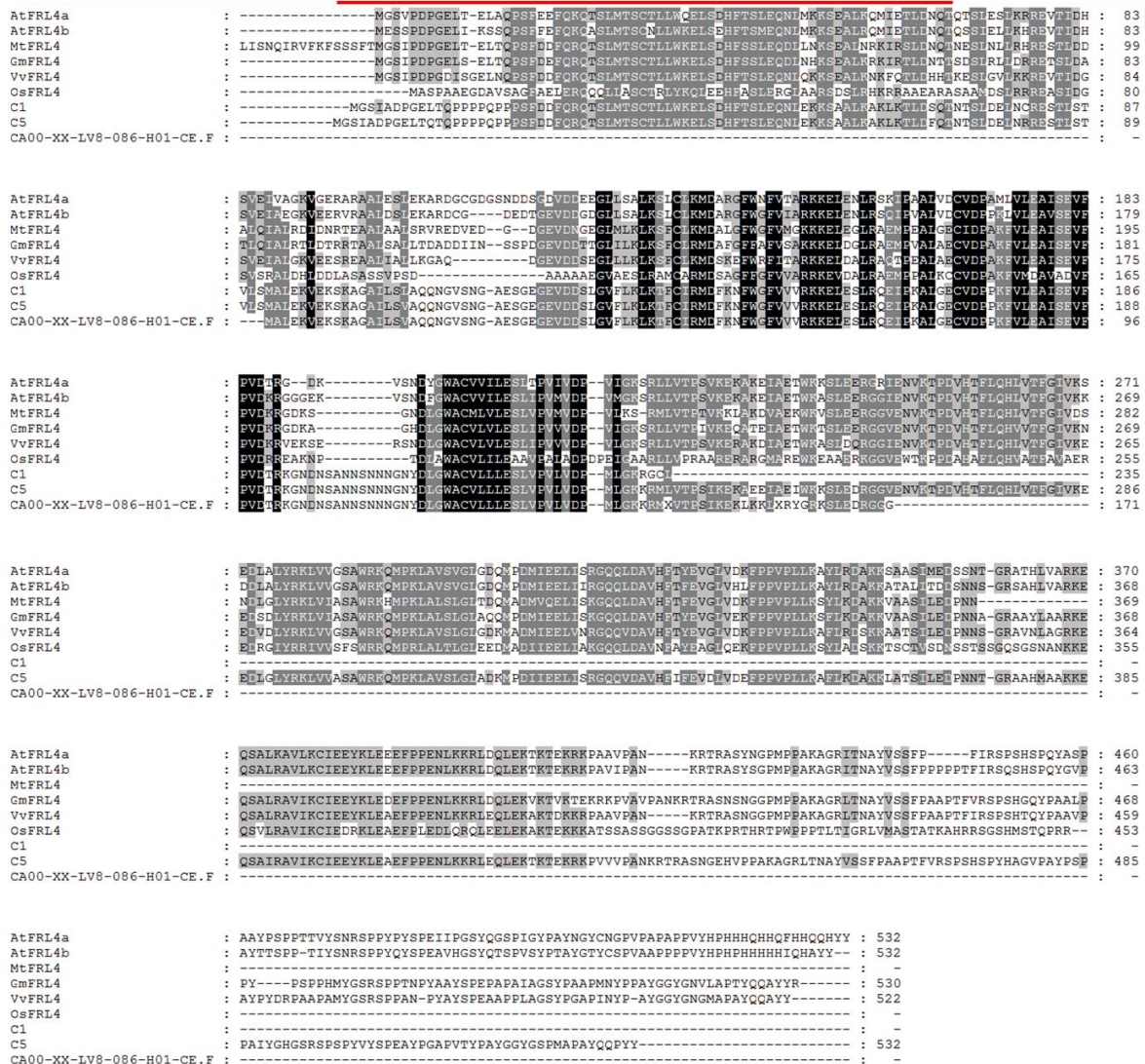
Relative expression through RT-qPCR

The relative expression pattern of *CaFRL4* is represented in figure 4, where one can observe that *CaFRL4* highest expression levels could be observed in root and leaf tissues. According to Risk et al., (2010), *FRL4* shows the highest expression levels among the other genes present in the *FRIGIDA* superfamily, with elevated expression in all plant tissues. These results can be proved in figure 4, where *CaFRL4* was expressed in all tissues analyzed in this study. The expression of *CaFRL4* in leaves during coffee flowering is relevant, since it can be related to the with

the regulatory action that *FRIGIDA* gene exerts upon *FLC*. Barreto et al., (2012) analyzed the expression pattern of *CaFLC* in coffee trees and observed that the highest expression level of this gene occurred in leaf tissue before flowering, from plants submitted to 90 days of water suspension, with its expression around four times lower when compared to leaf tissue during flowering. The similarity in the expression pattern of *CaFRL4* and *CaFLC* in leaf tissue during flowering can indicate a positive regulation of *FRL4* over *FLC*, since the decrease in the expression of these genes triggers the flowering process (CHAO et al., 2013; LIU et al.,

2016). In addition, different studies have shown that *FRI* genes are essential in the flowering regulation process, since when the variation in the gene sequence is accessed or when these genes are deleted, modifications in flowering time is observed.

Figure 3. *In silico* expression profile of putative coffee *FRL4* genes, the EST-*contig1* (C1), the EST-*contig5* (C5), and the *singlet* CA00-XX-LV8-086-H01-CE.F, found in the CAFEST database. The normalized number of *reads* for the transcripts in each library is represented by *grayscale*, where the darker the shade, the higher is the expression. Coffee libraries are as follows (Vieira et al. 2006): *AR1/LP1* plantlets and leaves treated with araquidonic acid, *BP1* suspension cells treated with acibenzolar-*S*-methyl, *CB1* suspension cells treated with aciben- zolar-*S*-methyl and brassinosteroids, *CS1* suspension cells treated with NaCl, *EA1/ IA1/IA2* embryogenic calli, *EM1/SI3* germinating seeds (whole seed and zygotic embryos), *FB1/FB2/FB4* flower buds in different devel- opmental stages, *FR1/FR2* flower buds + pinhead fruits + fruits at different stages, *CA1/IC1/PC1* non embryogenic calli with and with- out 2,4 D, *LV4/LV5* young leaves from orthotropic branch, *LV8/LV9* mature leaves from plagiotropic branches, *RM1* leaves infected with leaf miner and coffee leaf rust, *RT8* suspension cells with stressed with aluminum, *RX1* stems infected with *Xylella* spp.. The arrow indicates the EST-*contig* selected for RT-qPCR.
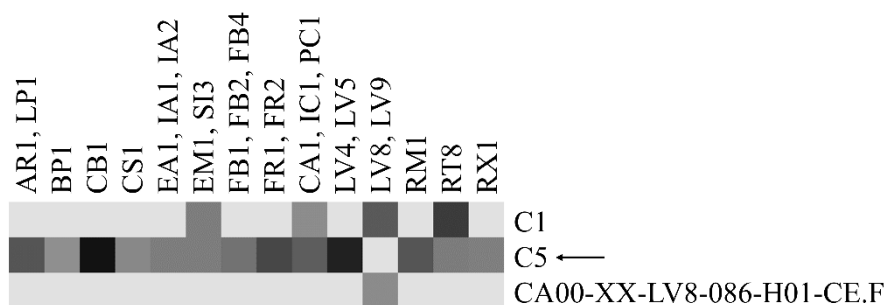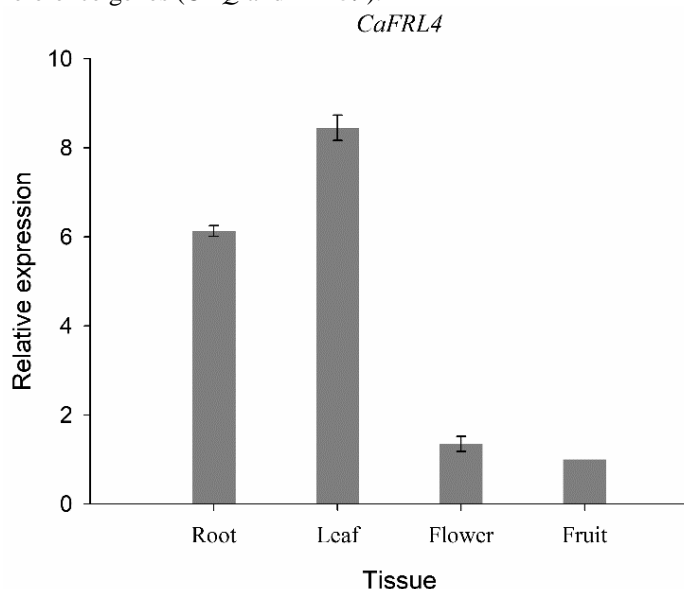

Figure 4. Relative quantitative expression profiling of *CaFRL4* in root, leaf, flower and fruit tissue. Columns represent the fold difference in gene expression for the tissues analyzed, relative to calibrator sample (Fruit tissue). Expression values in each tissue were obtained from three biological replicates and *error bars* represent the standard errors for them. Gene transcripts were normalized by two reference genes (*UBQ* and *RPL39*).

genes involved in the regulation of flowering in Coffee arabica. Additional studies of genetic transformation in model species will permit a better comprehension of the regulatory processes, as well as the identification of the exact function of this gene in flower induction, considering that it has not been functionally characterized yet.

The authors declare no conflict of interest.

CONCLUSION

This study enabled the identification and characterization of *CaFRL4*, which is one of the main

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