Prospection of Tissue Specific Promoters in Coffee

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SUMMARY

The majority of transgenic organisms reported in the literature have been made using constitutive promoters. However, there are economic, environmental and biosecurity related restrictions involving indiscriminate (constitutive) expression of heterologous genes. The usage of tissue-specific and induced promoters can resolve those issues by limiting the expression of a transgene to the necessary tissues and conditions. The promoters currently used at Embrapa are transnational properties, burdening the research and causing technological dependence. Therefore, the objective of this work was to find and characterize tissue and organ specific promoter in Coffea spp. We have used the Coffee genome database *in silico* tools to find genes preferentially expressed in root, leaf and fruit. In this way we found 72 organ-specific candidates: 18 apparently preferentially expressed on leaves, 14 on roots and 40 on fruits. Some of those candidates were tested *in vitro* using RT-PCR, semi-quantitative PCR, northern blotting and qPCR assays. All four leaf-specific candidates tested (GCFo1, GCFo2, GCFo3 and GCFo4) and at least one of the two fruit-specific candidates tested (GCFr1 e GCFr2) where confirmed to be preferentially expressed on their respective organs. Temporal and spatial expression assays showed that GCFr2 has its expression peak at the endosperm, 180 days after flowering. The highest expressed genes of leaf (GCFo3 and GCFo4) and fruit (GCFr2) were used as probes to isolate its respective promoter through a BAC libraries screening or using the Genome Walker Universal Kit (Clontech). Results concerning gene expression and the molecular characterization of these genes will be presented.

INTRODUCTION

Coffee culture is facing several problems that can drastically compromise its production in Brazil. In this scenario, the Brazilian Coffee Genome Project had the intention to supply information on coffee genome aiming at developing improved varieties. Once *Coffea arabica*, the most important variety, is perennial, tetraploid and has low genetic variability, the transgenia appears like a good way to obtain plants more suitable to different purposes. In fact, there are some established protocols of coffee transformation especially in *C. canephora* (Ribas et al., 2006) and promising experiences with *C. arabica* (Alpizar et al., 2008). Despite this, transgenics in general undergoes a lot of restrictions in part because of the indiscriminated expression of the transgene guided by constitutive promoters like 35S. The transgene expression in an organ-specific or condition specific manner is required for plant molecular breeding and could be addressed using adequate promoters (Wally et al, 2008 and Marraccini et al., 2002). With the aim to identify organ specific genes (leaf, roots and fruit)

that could be used as a probe to isolate its respective promoters, we performed an *in silico* analysis using the data basis of the Coffee Genome Project (Vieira et al., 2006). Our electronic northern has identified 18 leaf genes, 14 roots genes and 40 fruit genes. Some of these genes were assayed through RT-PCR, RT qPCR and northern blot approaches. In this way we confirm the organ preferentially expression of 4 genes of leaf, here named GCFo1, GCFo2, GCFo3 and GCFo4 and 1 gene of fruit, the GCFr2. Nowadays we are isolating its respective promoters to test then in model plants and, in the future, in coffee.

MATERIALS AND METHODS

In silico analysis

Based on the UniGene coffee databank (Vieira et al., 2006), ESTs libraries of only one tissue (leave, root or fruit) were grouped and contrasted against another group containing all the other libraries through an Accurate Fisher Test. The genes witch presented preferential expression in roots, leaves or fruit were selected. Among these were chosen those stronger and unpublished.

Expression analysis

Northern blot

Total RNA from *Coffea arabica* root, leaf and fruit were hybridized in SSPE to probes corresponding to partial sequence of the candidate genes labeled with ^{32}P dCTP.

RT Real Time PCR

RNA from root, leaf and fruit was treated with DNase and converted to cDNA. The PCR reaction was prepared using SYBR Green and specific primers and performed in a 7500 PCR systems (Applied Biosystems) as described by Bustin (2002). Normalization was done against ubiquitin gene.

Promoter isolation

Genome Walker approach

Specific reverse primers were designed at the 5´ sequence of the leaf candidates to amplify the 5´ promoter region from genomic DNA template by genome-walking method (Genome Walker Universal Kit, Clontech) following manufacturers protocols.

Screening of BAC libraries

Nitrocellulose membranes containing 18.432 genomic fragments of high molecular weight cloned in BACs were hybridized with the GCFr1 probe. Positive clone was digested with several restriction enzymes, subcloned and sequenced for promoter region isolation.

The promoters sequences obtained through the strategies described above were analyzed in PLACE database (http://www.dna.affrc.go.Jp/htdocs/PLACE/, Higo et al., 1999) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantCARE, Prestridge,1991) for identification of cis-elements

RESULTS AND DISCUSSION

The electronic northern blot analysis has revealed 103 UniGene tissue-specific. Among these, 18 refer to leaves, 40 to fruits, 14 to root and 31 to floral buttons. Those, 84% are homologous to known sequences and 16% are unknown. The first step to confirm the tissue specificity expression of candidate genes identified in the virtual analysis was a qualitative RT-PCR study, performed with total RNA samples extracted from leaves, roots and fruit and converted to cDNA. RT-PCR results showed that GCFo1, GCFo2, GCFo3 and GCFo4 transcripts are detected preferentially in leaves and GCFr1 was detected preferentially in fruit (Figure 1).

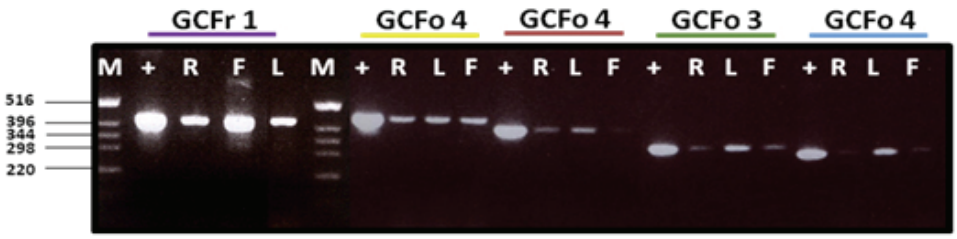

Figure 1. RT-PCR analysis for GCFo1, GCFo2, GCFo3 and GCFo4. Templates used in the reactions: (+) positive control, (R) root cDNA; (L) leaf cDNA; (F) fruit cDNA.

A quantitative analysis was then performed in order to access the expression ratio of these genes at each tissue. Data presented here are based in a relative quantification in comparison with the constitutive ubiquitine gene. The preferential expression in the target tissue was confirmed (Figure 2).

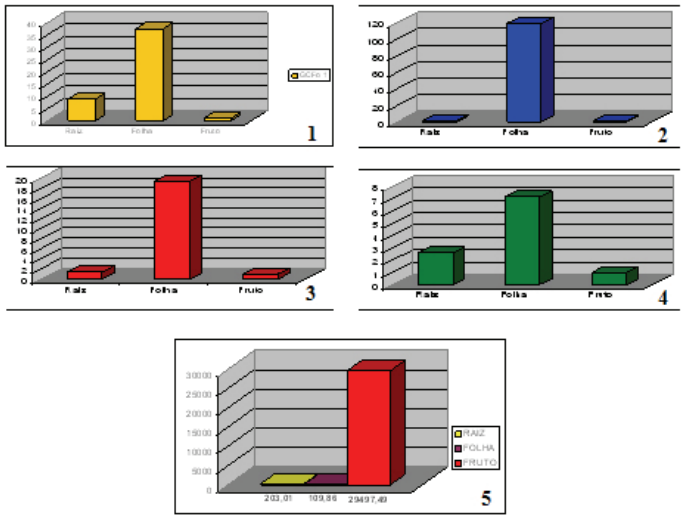

Figure 2. RT Real Time PCR relative analysis. GCFo1 (1), GCFo2 (2) GCFo3 (3), GCFo4 (4) and GCFr1 (5). Ubiquitine gene has been used as a constitutive control.

For the initial promoters isolation experiments the GCFo4 in the case of leaves and the GCFr1 in the case of fruit were chosen. The criteria adopted were: high expression level, the ineditism and the tissue specificity trait. In order to isolate these 2 promoters, two different approaches were used: genome walker method (GCFo4) and screening of BAC libraries (GCFr1) (Figure 3). The physical clones were obtained, sequenced and analyzed (Figure 4). In the case of GCFo4 promoter, sequence analysis by the PlantCare and PLACE showed the basic cis elements like TATA box and CAT box and, beside, putative elements responsive to

dehydration stress (ABRELATERD1) (Simpson et al., 2003), recognition sequence of *Arabidopsis* Athb-1 protein, which is characterized by the presence of a homeodomain (HD) with a closely linked leucine zipper motif (Zip) (CAATWATTG) (Sessa et al., 2003).

Our results show that the suggested strategy to isolate specific promoters is efficient. Expression assays *in vivo* are necessary to confirm the tissue specificity of the isolated promoters.

Once validated these promoters could be very useful to obtain transgenics to different conditions like drought, pathogen resistance and cup quality. These promoters could be assayed in other species to evaluate its performance in a universal way. The development of new promoters with tissue specific expression patterns is important for the future development of crops.

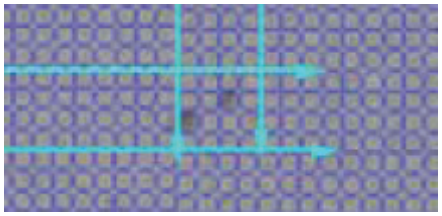

Figure 3. BAC libraries screening through hybridization with GCFr1 probe. Positive clones are delimited by green arrows.

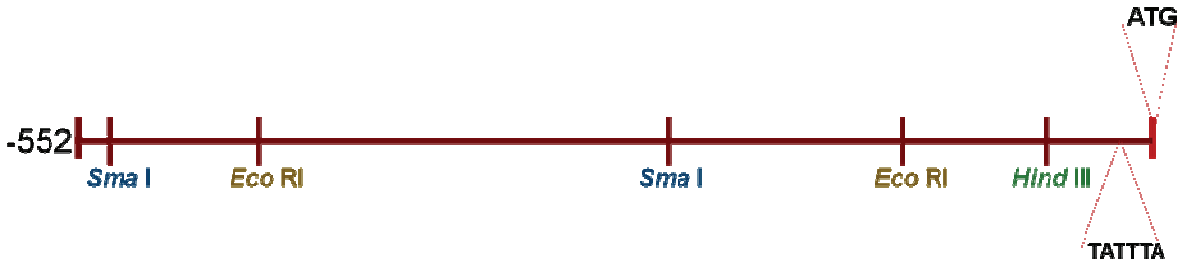

Figure 4. GCFo4 promoter restriction map.

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