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Gene expression analysis associated with tissue-specific promoters in *Musa* spp.

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ABSTRACT. The study of promoters has become essential to elucidate genetic regulation and allow new genetic transformation strategies through plant biotechnology. The challenge is to discover and validate promoters that can regulate gene transcription spatially and/or temporally. The goal of this work was to validate genes associated with tissue-specific promoters of bananas obtained from *in silico* sequences and selected from the DATAMusa databank. Gene expression was quantified using RT-qPCR from different tissues: leaves, flowers, roots, unripe pulp, ripe pulp, unripe peels, and ripe peels of two different genetic groups: Prata-Anã (PA; group AAB) and Grand Naine (GN; group AAA). After the analysis of the expression of genes associated with the promoters, normalization was performed with the most stable reference genes (*TUB* and *L2*) selected using the RefFinder tool. It was determined that five genes were specific or expressed to a greater extent in some tissues than others. The EMB-23 gene was highly expressed in ripe pulp and flowers of GN, EMB-26 in the ripe pulp of GN, EMB-27 in flowers of GN, EMB-28 in roots of PA and ripe pulp and roots of GN, and EMB-31 in roots and flowers of GN and PA, and unripe pulp of GN. The *in silico* analysis was efficient in the identification of spatial/time-specific genes, thereby decreasing analysis time and cost, making future genetic transformation studies focusing on the application of these tissue-specific promoters possible.

Keywords: banana; RTqPCR; plant biotechnology; gene expression; genetic transformation.

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Introduction

Promoters are specific DNA sequences close to the transcription starting point (TSP) that allow RNA polymerase positioning as soon as transcription factors recognize them. Plant promoters can be characterized based on different functional aspects, such as biotic or abiotic stress-inducible, light-responsive, chemical-inducible, hormone-inducible, constitutive, and tissue-specific (Dey, Sarkar, Acharya, & Maiti, 2015).

Great advances have been made with the use of well-known and characterized promoters, such as the 35S promoter from the cauliflower mosaic virus, the promoter of the encoding corn polyubiquitin gene (Ubi-1), and the promoters of the genes involved in the synthesis of opines (octopine and nopaline) of *Agrobacterium tumefaciens* (Dale, Paul, Dugdale, & Harding, 2017). Nevertheless, genes regulated by constitutive promoters have been expressed in all organs and/or tissues during transformed plant growth, with low control of protein expression, which could be disadvantageous (Freeman, Sparks, West, Shewry, & Jones, 2011).

For plant breeding programs, it is important to include promoters that specifically assure spatial- or temporal-specific gene expression. The use of space-time or tissue-specific promoters capable of achieving more direct and precise regulation is described in the literature. In *Populus*, the Potri.013G007900.1 promoter (known as PtrMX3) is functional only in mature xylem, hence being useful for biotechnological applications, which aims to improve the physicochemical properties of wood (Nguyen et al., 2016). The barley Hvhsp17 gene promoter can be induced by heat treatment in transgenic wheat. This heat-inducible promoter makes it possible to investigate the function of candidate genes by overexpression or downregulation of the target gene expression (Freeman et al., 2011).

Studies related to prospection and analysis of promoters with specific organ/tissue expression contribute to the elucidation of the mechanisms of transcription regulation, as well as other molecular processes, such as overexpression or knockdown of target genes for specific tissues and/or specific growth stages (Freeman et al., 2011). The use of promoters obtained from the same target species could lead to increased social approval related to the production and commercialization of genetically modified plants (GMPs), whose transgene expression does not present involuntary pleiotropic side effects, which often follow when the constitutive 35S promoter is used (Nguyen et al., 2016).

This scope is particularly advantageous for bananas (*Musa* spp.), whose leaves and roots are frequently attacked by the most aggressive plant pathogens, causing diseases such as Black or Yellow Sigatoka and Panama disease (PROMUSA, 2020). Furthermore, it is important to remember that banana plants are sterile; in other words, they do not pass their genes onto other plants and are easily cultivated in tropical countries. Furthermore, they could provide a good option for a vaccine or drug delivery vehicle (Bhairy & Hirlekar, 2017).

Therefore, GMPs for pathogen-resistance with gene expression driven by a cisgenic tissue-specific promoter for leaf or root would aggregate sustainability to the culture against the pathogens, thereby becoming more acceptable to consumers. Furthermore, the expanding cultivation of bananas observed in several countries (FAO, 2020) has increased the technological dependence of the crop, subjecting it to biotechnological applications, more specifically to genetic transformation or even gene editing. Therefore, classic breeding is limited because of polyploidy, parthenocarpy, and the long-life cycle of commercial varieties. Thus, new molecular tools (such as the prospection of tissue/time-specific promoters) could enable the production of improved plants.

The goal of the current study is to prospect banana tissue-specific promoters based on 27 gene sequences expressed *in silico* and collected from the DATAMusa databank.

Material and methods

Plant material

Different plant tissues taken from banana plants were used: leaves, flowers, roots, unripe and ripe pulp, and unripe and ripe peels of Prata-anã (genomic AAB group; Prata sub-group) [PA] and Grand Naine (genomic AAA group; Cavendish sub-group) [GN]. Leaves, flowers, roots, and unripe fruit tissues of both cultivars were collected from the experimental field at Lavras Federal University - UFLA (21°14′42″ S, 45°00′00″ W), washed in distilled water, frozen in liquid N_2, transported to the laboratory, and stored at -80°C. Some unripe fruits of both cultivars were stored, and later their ripened peels and pulps were collected and immediately frozen in liquid N_2 and stored at -80°C until RNA extraction.

In silico sequences selection

Fifty-five candidate gene sequences expressed *in silico* were previously selected from the DATAMusa databank (Souza Júnior et al., 2005). These genes were analyzed with BLASTn to check function, and where trunked genes, unknown proteins, or transposons were detected, the genes were eliminated. For the remaining genes, the 2 kb upstream sequence for each predicted gene in the Rice Genome Automated Annotation System (RiceGAAS) was used for the analysis. For confidentiality reasons, these sequences were numbered from EMB-1 to EMB-55.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), dissolved in RNase-free water, and stored at −80°C. RNA integrity was determined by denaturing agarose gel (1.2%) electrophoresis in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]) and stained with ethidium bromide (EtBr). The intense ribosomal RNA bands without smears after electrophoresis confirmed the RNA integrity. The RNA concentration of each sample was measured in triplicate by using a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). The RNA purity was measured using the 260/280 nm ratio, with expected values between 1.8 and 2.0.

Thereafter, the RNA samples were treated with Turbo DNase (Ambion, Waltham, MA, US), according to the manufacturer's recommendations. cDNAs were synthesized from 1 µg of total RNA in triplicate by using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher, Waltham, MA, USA) following the manufacturer's recommendations. The cDNA quality was confirmed by *TUB* gene amplification followed by 1.0% agarose gel electrophoresis using 10 µL of the PCR product. The synthesized cDNAs were stored at −80°C for further RT-qPCR.

Selection of reference genes and primer design of candidate genes

A set of four reference genes was selected, including several commonly used reference genes for bananas (Chen et al., 2011; Podevin, Krauss, Henry, Swennen, & Remy, 2012; Zhang, Missihoun, & Bartels, 2017), such as actin (*ACT1*), tubulin (*TUB*), elongation factor 1α (*EF-1α*), and ribosomal protein L2 (*L2*). Orthologous sequences were searched for reference genes using the Basic Local Alignment Search Tool (BLAST) and the available sequences for the genes under study from the GenBank (https://www.ncbi.nlm.nih.gov/) and DATAMusa databank.

Primer pairs used for the 27 *in silico*-selected sequences were designed using the Primer Express v.3.0 (Applied Biosystems) program (sequences not available due to commercial protection). The length of the primers was set between 20 and 22 bp with a GC content ranging from 45% to 60% and a melting temperature (Tm value) in the range 55 - 65°C. Primer pair specificity was verified through a dissociation (melting) curve analysis. Both PCR amplification efficiency (E) and the regression coefficient (R^2) were determined during the validation of primers according to the standard curve method using a set of all cDNA samples with 5× serial dilutions.

RT-qPCR amplification

RT-qPCR analyses were performed using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, USA) with a reaction mix containing 5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, USA), 10 ng of cDNA template, 10 ng of primers for the gene, and RNase-free water for a total volume of 10 µL. Amplification conditions were as follows: initial enzyme activation at 95°C for 10 min. and 40 cycles of denaturation at 95°C for 15 s with both annealing and extension steps at 60°C for 1 min. To confirm the primer specificity, melting curves were recorded after the 40 amplification cycles were completed by increasing the temperature from 60 to 95°C. All qPCR assays were conducted in technical and biological triplicates.

Expression stability analysis of candidate genes

The expression levels of candidate genes were determined based on the quantification cycle (Cq), also known as the threshold cycle (Ct), being defined as the cycle during which the fluorescence from amplification exceeds that from the background (Kozera & Rapacz, 2013). The Cq values were determined using the 7500 software version 2.0.5 (Applied Biosystems, USA) and corrected according to the efficiency of each primer pair (Freitas et al., 2017).

Expression data were quantified using the − ΔΔCT method (Livak & Schmittgen, 2001). Normalization was conducted with the expression of the most stable genes selected by the RefFinder (http://150.216.56.64/referencegene.php) tool among *ACT1*, *TUB*, *EF-1α*, and *L2* reference genes. Box plot diagrams were created using Microsoft Excel 2013 to illustrate the level and variation in expression of each tested reference gene.

The RefFinder tool (https://www.heartcure.com.au/reffinder/) was employed to assess the gene expression stability in the cDNA pool of all tissues (leaves, flowers, roots, unripe and ripe pulp, and unripe and ripe peels of PA and GN).

Results and discussion

Specificity and efficiency of reference genes

Primer specificity during PCR amplification was confirmed by the presence of one peak on the dissociation curve (melting curve). The PCR efficiency (E) and regression coefficient (R^2) were calculated using the standard curve inclination determined for each primer pair. E-values ranged from 98% to 99.7% and R^2 displayed values were $\geqslant 0.8483$ (Table 1); thus, indicating that at the end of each cycle the cDNA mold was successfully doubled.

Stability of reference genes expression

To indicate the stability of reference gene expression, the programs geNorm (Vandesompele et al., 2002), NormFinder (Andersen, Jensen, & Ørntoft, 2004), Bestkeeper (Pfaffl, Tichopád, Prgomet, & Neuvians, 2004), and Delta-Ct (Silver, Best, Jiang, & Thein, 2006) were used with the RefFinder tool. This tool generates an analysis of each program and makes a general comparison and classification of candidate genes tested, thereby decreasing contrasting result differences (Freitas et al., 2017).

In the pooled samples, an expression stability analysis of the reference genes indicated average expression stability values (*M*) (through geNorm for the most stable genes, *L2* and *TUB* ($M = 1.075$), and for the least stable genes, *EF-1* ($M = 1.539$) and *ACT* ($M = 2.510$) (Table 2).

Table 1. Description of the candidate reference genes for RT-qPCR analysis.

Gene code	Accession number	Primer sequence (forward/reverse 5'–3')	Tm (°C)	E (%)	R^2
ACT1	EH413666	5´CCCAAGGCAAACCGAGAGAAG 3´ 5´GTGGCTCACACCATCACCAG 3´	61	98.0	84.83
TUB	JQ744274.1	5´TGTTGCATCCTGGTACTGCT 3´ 5´GGCTTTCTTGCACTGGTACAC 3´	58	99.7	96.84
EF-1	EH413178.1	5´CGGAGCGTGAAAGAGGAAT 3´ 5´ACCAGCTTCAAAACCACCAG 3´	55	99.25	86.92
L2	EH413647	5´AGGGTTCATAGCCACACCAC 3´ 5´CCGAACTGAGAAGCCCCTAC 3´	59	98.65	93.91

Table 2. Ranking of candidate reference genes according to their stability values obtained via geNorm, NormFinder, BestKeeper, and Delta-Ct algorithms.

Method	Ranking Order			
	1	2	3	4
Delta-Ct	*TUB* (1.938)	*L2* (2.239)	*IF-1* (2.382)	*ACT* (3.48)
BestKeeper	*IF-1* (1.564)	*TUB* (1.625)	*L2* (1.736)	*ACT* (1.969)
NormFinder	*TUB* (0.537)	*L2* (1.304)	*IF-1* (1.549)	*ACT* (3.294)
GeNorm	*L2/TUB* (1.075)		*IF-1* (1.539)	*ACT* (2.51)

The sample pool comprised leaves, flowers, roots, unripe and ripe pulp, and unripe and ripe peels of PA and GN (*Musa* spp.). Actin (*ACT1*), tubulin (*TUB*), elongation factor 1α (*EF-1α*), and ribosomal protein L2 (*L2*).

Using the NormFinder program analysis, it was determined that *TUB* (Stable Value (SV) = 0.537) was the most stable reference gene, whereas *ACT* (SV = 3.294) was the least. However, through the BestKeeper program analysis, the most stable reference gene was *EF-1α* (DP = 1.564), which was different from the NormFinder results; however, it confirmed that the least stable reference gene was *ACT* (DP = 1.069). As determined by the Delta-Ct method, *TUB,* followed by *L2,* were the most stable genes, with stability values of stability ΔCt value (ΔCt) 1.938 and ΔCt 2.239, respectively. *ACT* was the least stable gene (ΔCt = 3.48), according to all other analyses. The final ranking suggested that the most stable reference gene was *TUB* (overall stability value [OSV] = 1.189) followed by *L2* (OSV = 1.861)*,* whereas *ACT* was the least stable (OSV = 4.0) (Figure 1).

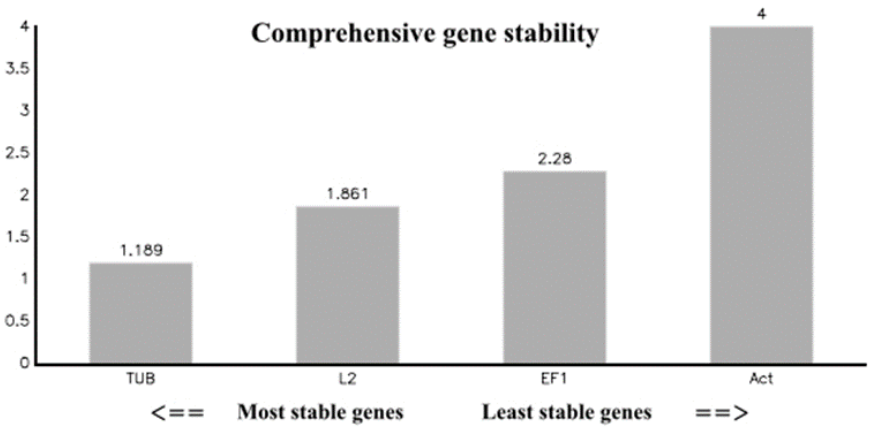

Figure 1. The final rankings through the RefFinder tool.

These data were similar to those obtained for dwarf banana seedlings of the cultivar Guangfen (*Musa* spp. ABB) inoculated with *Bacillus subtilis strain TR21* and *Fusarium oxysporum f. sp. cubense,* for which it was also determined that *L2* and *TUB* were the most stable reference genes (Zhang et al., 2017). However, as shown by other groups, the most frequently used reference genes are not always the best. The reference genes *ACT* and *GAPDH* were less stable in several banana samples (*Musa acuminata* AAA Group, Cavendish), including freshly harvested roots, leaves, flowers, and pre-climacteric bananas (Chen et al., 2011). The selection of stable reference genes for different types of plant tissues is an essential step to obtain reliable and accurate results of target gene expression through RT-qPCR (Freitas et al., 2017).

Promoters validation through tissue-specific gene expression analyses

Gene expression of the sequences selected in the DATAMusa databank as being tissue-specific was quantified through RT-qPCR using the cDNAs obtained from the seven tissues of the two banana cultivars described in the Materials and Methods section. It is important to note that tissue-specific genes (promoters) are expressed almost exclusively in one target tissue (Gurr & Rushton, 2005). In this study, from the 27 analyzed gene sequences, the genes named EMB-23, EMB-26, EMB-27, EMB-28, and EMB-31 demonstrated significant expression in only one target tissue (Figure 2) or two tissues but they were not present at the same time (temporal expression).

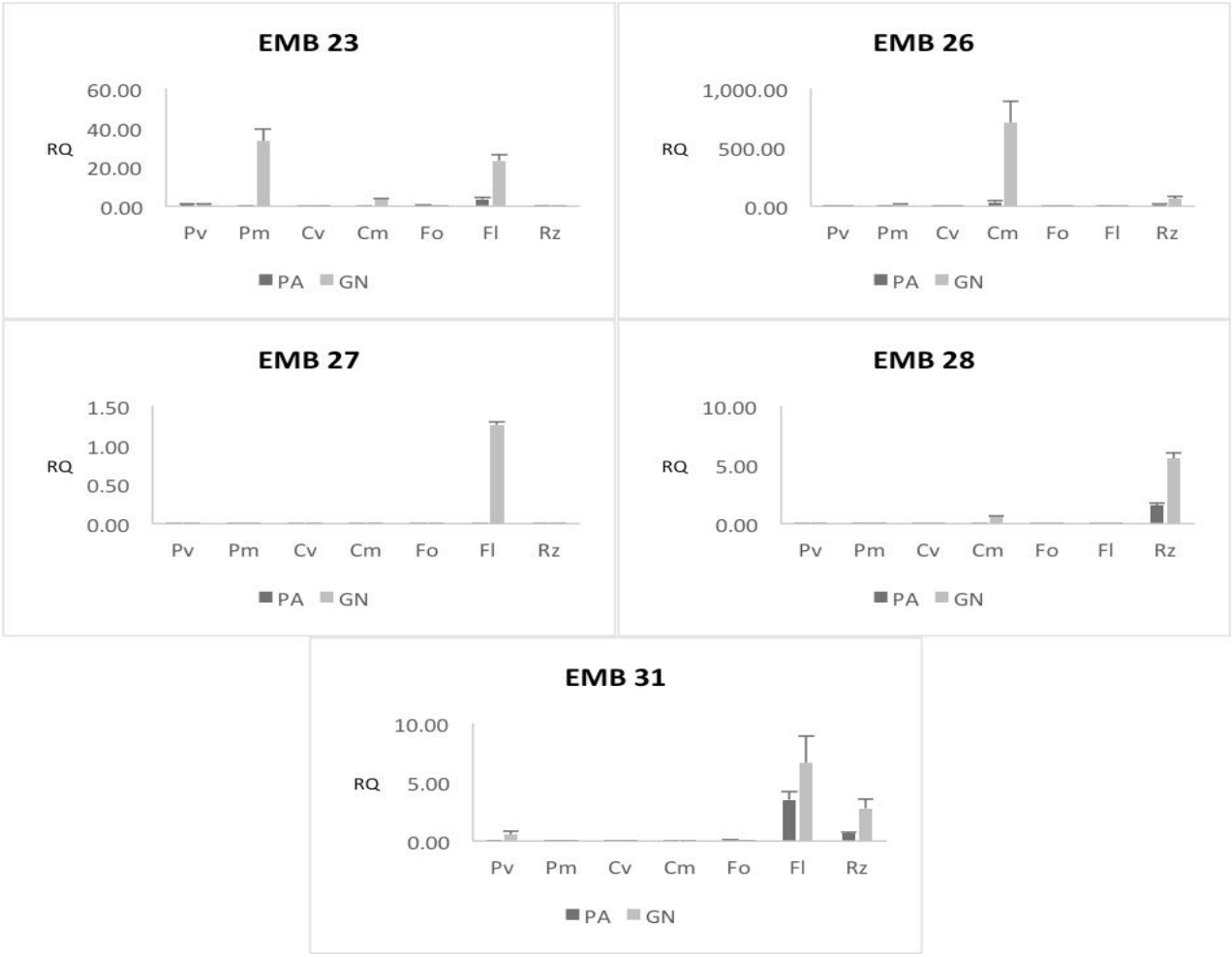

Figure 2. Relative expression (RQ) of EMB-23, EMB-26, EMB-27, EMB-28, and EMB-31 sequences among the 27 analyzed sequences through RT-qPCR. The columns represent the expression in tissues: unripe pulp (Up) ripe pulp (Rv), unripe peels (Up), ripe peels (Rp), leaves (L), flowers (F), and roots (R) of Prata-anã (PA, dark gray) and Grand Naine (GN, light gray).

Our *in silico* analysis of the sequences confirmed the function of EMB-23, EMB-26, EMB-27, EMB-28, and EMB-31 genes corresponding to 18.2 kDa class I heat shock, cytochrome P450-71D10 mitochondrial enzyme, reductase aldehyde enzyme, cationic peroxidase precursor, and a protein associated with anaphases, respectively.

A higher expression of the EMB-23 gene (18.2 kDa class I heat shock) was found in GN ripe pulp (Figure 2A). Some authors described the ripening process as the final stage of maturation and the beginning of senescence, considering it a process with many cellular texture/structure transformations and plant stress. This involves the dismantling of multiple polysaccharide networks by diverse families of cell-wall-modifying proteins, including enzymes for pectin and cellulose catabolism (Wu et al., 2014). Therefore, a higher expression of proteins induced by stress may occur during the ripening process. Furthermore, EMB-23 gene expression was also present in flower tissue; however, it is possible to state that this gene is associated with a temporal tissue-specific promoter once the ripe pulp can be seen after fruit development and ripening and that the EMB-23 gene was not expressed in the unripe pulp, suggesting the usefulness of this gene at both developmental stages.

The 18.2 kDa class I heat shock protein, which belongs to the "small heat shock" (HSP) genic family, is related to the response to different types of stress, such as cold, wounding, pathogen infection, oxidative stress, and salicylic acid and methyl jasmonate treatment (Zhang et al., 2009). Oxidative stress, such as that caused by free radicals produced during ripening and senescence (Huan et al., 2016; Rosalie, Léchaudel, Dhuique-Mayer, Dufossé, & Joas, 2018), is linked to the action of P450 complex enzymes that play a fundamental role in oxygen activation and the generation of oxygen-reactive species [ORS] (Kim et al., 2022).

Higher expression of the EMB-26 gene (P450-71D10 cytochrome enzyme) was found in the GN/PA ripe peel. The cytochrome P450 monooxygenase superfamily (P450s) catalyzes a wide variety of monooxygenation reactions in primary and secondary metabolism in plants. As a result, numerous secondary metabolites function as growth and developmental signals or protect plants from various biotic and abiotic stresses. Moreover, the *P450* gene is closely involved in the regulation of plant hormone metabolism and directly functions in plant growth and development. Hormones are also involved in the formation of flowers, stems, leaves, the shedding of leaves, and the development and ripening of fruits (Xu, Wang, & Guo, 2015).

Therefore, the relationship between the two proteins mentioned above and the presence of ripe tissues explains the increase in gene expression. These results corroborate those of studies that showed an increase in proteins belonging to the families of small HSP, 17.3kDa class II HSP, cytochrome c reductase-processing peptidase subunit II, which, after ethylene treatment, may effectively contribute to the banana peel ripening process (Toledo et al., 2012; Du et al., 2016).

Higher expression of a protein from the aldehyde dehydrogenase (ALDH) reductase (EMB-27) superfamily was observed in GN banana flower tissue (Figure 2C). Proteins from this family have a conserved primary structure and have been identified as being responsive to different abiotic stresses, probably to function as "kidnappers" of reactive aldehydes generated during lipid peroxidation (Zhao et al., 2017). These proteins use either NAD^+ or $NADP^+$ as cofactors to perform reactions that produce intermediates from several catabolic and biosynthetic pathways. NADH/NADPH molecules are a major source of reducing equivalents required for maintaining cellular redox balance (Brocker et al., 2013). Furthermore, the expression of a gene from the ALDH superfamily was associated with the aroma of rice lines, such as jasmine and basmati (Sakthivel, Sundaram, Rani, Balachandran, & Neeraja, 2009), and the same study showed that rice aroma quality is influenced by the tilling area temperature, as shown by the differential expression of the *Badh2* gene (belonging to the ALDH superfamily), and the resulting formation of the volatile 2-acetyl-1-pyrroline (2AP) compound (Prodhan, Faruq, Taha, & Rashid, 2017). Therefore, detecting a tissue-specific gene (flower) promoter has become of great interest to both species with non-homogenous flowering, such as *Coffea arabica* (Oliveira, Cesarino, Mazzafera, & Dornelas, 2014), and the growth of flowers with differentiated colors, as reported in other studies (Miyagawa et al., 2015).

For the EMB-28 gene, a higher tissue-specific expression was observed in GN roots (Figure 2D). This gene induces the translation of a cationic peroxidase precursor, and it is mostly expressed in the roots because it is suitable for oxidative stress protection (Kumaravel et al., 2016). Class III cationic peroxidases are found in the extracellular space or even in plant vacuoles and are involved in hydrogen peroxide degradation related to stress responses and auxin catabolism, as well as in lignin and flavonoid biosynthesis (Dubrovskaya et al., 2017). Embryogenic callus induction in banana plants is associated with the ability to control oxidative stress, thereby regulating the ROS elimination system through the peroxidase cationic enzyme. This enzyme was most abundant in embryogenic cell proteomes when compared to non-embryogenic cells (Kumaravel et al., 2016).

Finally, higher expression of proteins related to anaphase (EMB-31 gene) was found in GN flower and root tissues and PA roots (Figure 2E). This result could be associated with the high cell division rates in developmental stage tissues, as in the case of flowers or tissues under constant growth, such as the roots. At anaphase, the duplicated chromosome sister chromatids are split, then pulled by the shortening microtubules that are connected to kinetochores and by the degradation of tubulin molecules, which keep sister chromatids together (Watson et al., 2008). This important mitosis phase is mediated by the participation of many proteins, which ensures that each daughter cell receives an identical set of chromosomes.

The relevance of banana tissue-specific promoters cannot be restricted to being leaf and/or root specific, which could be used for sustainable pathogen control. Given the daily consumption of bananas, such relevance extends to pulp-specific promoters that might increase its nutritional value or even promote vaccine synthesis. Furthermore, many genes that play important roles in various metabolic and regulatory pathways in banana plants have already been reported. To use such genetic resources efficiently, specific

promoters that can drive gene expression in a highly controlled fashion are necessary (*i.e.,* specific expression regarding tissue growth), and they can also be used for gene editing.

Conclusion

The identification and characterization of five potential putative tissue-specific promoters are described. The EMB-28 gene expressed in the roots and any tissue other than the pulp is relevant in the production of cisgenic bananas, and to induce gene expression in roots for resistance to soil pathogens. Additionally, EMB-23, which is expressed mainly in ripe pulp, could be used for public health research. The EMB-26 gene promoter was expressed on ripe peels and may be used in breeding programs aimed at controlling fruit ripening and peel diseases. Moreover, EMB-27 and EMB-31 promoters may be applied not only to studies concerning flowering but also to support breeding programs of other monocots plants.

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