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MOLECULAR RESPONSE OF COCOA (*Theobroma cacao*) TO WATER DEFICIT CONDITIONS

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ABSTRACT

Theobroma cacao is very important in the economy of many tropical countries, especially in Ecuador. The development and production of *Theobroma cacao* have been limited by the attack of diseases, pests, and currently by the impact caused by the water deficit. The loss of water in the crop causes a low production of the plantations. In addition, when genetic diversity is scarce, the possible combinations of genes capable of conferring biological adaptation and, consequently, the capacity to adapt to environmental variations, are reduced. Therefore, a viable option is to identify genes capable of conferring adaptation to water deficit stress. The objective of this research was to characterize genes of *Theobroma cacao* in response to water deficit. The sequencing and characterization of genes related to water stress were achieved and, finally, the hybridization profile of the genes (ABA, ODC, DEH, and ABS) in response to Actin (ACT) was analyzed. The ABS gene hybridized 100 % with DNA from rootstock EET399 in roots and 80 % with DNA from graft T24 in leaves. The OCD gene hybridized 80 % with DNA from rootstock IMC 67 and 25 % with the T800 graft in leaves. The DEH gene hybridized below the normalizing gene (ACT) in both rootstock EET400 (10 %) in root DNA and graft T12 (5 %) in leaves. Finally, the ABA gene did not hybridize with the DNA of the CCN51 rootstock or with the graft (T801) in both roots and leaves, respectively. This research is key to understand some molecular mechanisms of *T. cacao* in response to water deficit and can be considered as a viable strategy to reduce crop agronomic losses under the current environmental scenario.

Keywords: Genes- Hybridization-Rootstock-Grafts,-Water deficit-Local Species

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INTRODUCTION

Cocoa (*Theobroma cacao*) is a tropical evergreen tree of commercial importance, cultivated for its almonds that are used in chocolate production (Carranza *et al*. 2020). The amount and distribution of rainfall result in reduced yield and vitality of cocoa trees in the long term (Läderach *et al.*, 2013). Therefore, rainfall tends to be the single most important factor influencing cocoa distribution (Asante *et al*., 2017). Cocoa grown in monocultures can be exposed to unfavorable conditions when temperature rises and humidity drops (Niether *et al*., 2018). Cocoa is highly susceptible to drought and the cropping pattern is related to rainfall distribution (Anim-kwapong and Frimpong, 2008).

The study of climatic elements is vital for the sustainable development of agriculture in a region (Grassi *et al*., 2017). In this context, plants have a wide range of responses according to the different alterations associated with stress due to biotic or abiotic factors, such as alterations in gene expression and cell metabolism, generating changes in growth and crop yield (Yepes and Silveira, 2011). Water limitation causes a significant

reduction in cocoa yield, coupled with genotypic variation is closely related to such deficit (Lahive *et al*., 2019). One response may be directly due to stress such as drought, which induces loss of membrane integrity, but resistance and sensitivity vary according to species and genotypes, as well as to the developmental stage, organ, and tissue type.

The changes that occur at the plant molecular level in response to abiotic stresses include changes in gene expression and cellular transduction signaling, which have been analyzed for many years (Ma *et al*., 2020). Likewise, several transcription factors and signaling molecules, which play an important role in cellular homeostasis under stress conditions, have also been studied (Buchanan *et al*. 2015). Plants are frequently affected by water stress and soil salinity, which are observed in many habitats. Plants have developed several strategies to cope with adverse environmental conditions. The adaptive and developmental mechanisms of plants allow them to survive adverse conditions or specific growth habitats to avoid stressful conditions.

In this context, stress-tolerant plants have involved certain adaptive mechanisms for different degrees of tolerance, which are largely determined by phenotypic plasticity. Several studies have been carried out to determine the effect of water deficit on cocoa growth and development. It has been shown that there are drought-tolerant cocoa genetic groups with high photosynthetic rate and stomatal conductance, along with low transpiration rate and thus higher water use efficiency (Apshara *et al*., 2013; Kunikullaya *et al*., 2018; 2002; ZakariyyaandIndradewa, 2018).

In recent years there has been a considerable increase in scientific and commercial interest in understanding the mechanisms by which plants respond to abiotic stresses. Whether by traditional breeding or genetic engineering, the objective is to manipulate these mechanisms and thus increase crop productivity in affected areas. This increase in productivity is intended as an alternative to avoid the expansion of the agricultural frontier, which is slowed by both urban growth and the protection of forests and protected areas (Buchanam, *et al.*, 2000). In addition, genetic diversity is considered an important factor in increasing productivity.

The magnitude and structure of the genetic diversity of a population determine its ability to adapt to the environment through natural selection. This is due to the fact that, when genetic diversity is low, the possible combinations of genes capable of conferring biological adaptation and, consequently, adaptability to variations in environmental conditions are reduced, which in turn reduces the probability of the emergence of new valuable individuals in the population. Thus, a population growing in the wild (or managed in a protected area) must have the adequate genetic diversity to ensure its existence in the face of constant changes in the biotic and abiotic components of its ecosystem. Based on the above, the objective of this work was to characterize *T. cacao* genes in response to water deficit, using roots and young leaves from different combinations of cocoa rootstocks and grafts from Ecuador as plant material.

MATERIALS AND METHODS

Location: The research was conducted at the Plant Biotechnology Laboratory of the Biotechnology Department of the Technical State University of Quevedo in Ecuador, from January to December 2018.

Experimental material: The plant material was selected from the Pichilingue Tropical Experimental Station of the National Institute of Agricultural Research (EETP-INIAP) of Ecuador, from an experimental nursery trial, under conditions of water deficit. Leaves and young roots of a combination of rootstocks (EET400, EET 399, CCN51, IMC 67) and grafts (T800, T801, T12, T24) were used as experimental material. At the time of collection, roots and leaves were washed, labeled, placed in liquid nitrogen, and then stored at -80°C until use.

Total RNA extraction and purification: For cocoa total RNA extraction, the protocol described by Morante *et al*. (2014) was used, to which modifications were included. 1) tissue weight of 100-300 to 500 mg, 2) addition of extraction buffer, 10 to 12 ml per gram tissue, 3) addition of extraction buffer, 10 to 12 ml per gram tissue, plus 2 % mercaptoethanol, 4) precipitation of RNA (with 10 M LiCl for four hours in cold). To eliminate the presence of genomic DNA, the DNase I enzyme from Invitrogen (USA) was used, which consists of the digestion of single and double-stranded DNA to oligodeoxyribonucleotides with phosphate at the 5' end, following the manufacturer's instructions.

cDNA synthesis and retrotranscription assay (RT): For cDNA synthesis, the "Super Script III First-Strand Synthesis for RT-PCR" reagent kit from Invitrogen (USA) was used, following the manufacturer's instructions. DNAse I-treated cocoa total RNA was used as a template for the retrotranscription (RT) assay. To this mixture the following combination of reagents were added: 10x RT Buffer, 25 mM MgCl2, 0.1 M DTT, RNase OUT, and Superscript III RT followed by incubation at 50 ºC for 50 minutes, 10 minutes at 25 ºC and 50 minutes at 50 ºC. The incubation phase was completed in 5 minutes at 85 ºC and then rested on ice for 1 minute. Finally, 1 µl of RNase H was added and incubated at 37 °C for 20 min. The synthesized cDNA was stored at -20 °C in aliquots for subsequent use in PCR amplification assays.

Oligonucleotide design: Five oligonucleotide pairs were designed from multiple alignments of highly conserved

sequences in which genes related to water deficit in plants have been identified, available at NCBI (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/). These genes were: ABA (abscisic acid), ODC (ornithine decarboxylase), ABS (abscisic stress), ACT (actin), DEHY (dehydrin). The oligonucleotides were tested in pairs and subsequently combined to increase the probability of hybridization, using genomic DNA and cDNA as templates. All oligonucleotides used in this work were synthesized and supplied by Sigma-Aldrich, USA. (Table 1).

DNA electrophoresis: Amplification products were separated and validated by horizontal DNA electrophoresis on 1 % (w/v) agarose gels with ethidium bromide staining. Gels were prepared in 1X TAE (tris, sodium acetate, EDTA) buffer containing: 40 mM Tris-base; 1 mM EDTA, 20 mM acetic acid. The gels were analyzed with a digital photo documenter (E-Gel Imager, Life Technologies) incorporating an ultraviolet UVP transilluminator and a high-resolution digital camera.

DNA purification and sequencing: The amplified DNA fragments separated by electrophoresis were cut out of the agarose gels using a scalpel, avoiding contamination between the bands. They were then purified with the "Purelink Quick Gel Extraction" kit from Invitrogen (USA), according to the manufacturer's instructions. Sequencing of the fragments of interest was carried out on a Li-cor sequencer by the Sanger method. DNA sequences were obtained by hybridization with the universal oligonucleotides M13 forward and M13 reverse. 1 µg of DNA was sequenced in a 15 µl volume of each sample.

Molecular characterization of genes: For the characterization of the coding genes related to water deficit, the Bio-Edit program (USA) was used to edit sequences and construct alignments. The BLASTX program (Altschul *et al*., 1990), which compares the percentage of similarity with other coding sequences for genes related to water deficit in *T. cacao* and other species, was used to compare the sequences with sequences from public databases. Sequence alignment was performed in the CLUSTALW program (Thompson *et al*., 1997) available online at the website of the European Bioinformatics Institute (www.ebi.ac.uk). For the search and comparison of sequences of interest, the NCBI public database (http://www.ncbi.nlm.nih.gov/) was accessed.

Hybridization profiles of genes on actin-agarose gels: For the analysis of the hybridization profile, PCR assays were performed for the ABA, ODC, ABS, DEHY, and ACT genes, using 30 ng of DNA as a template in all cases. Subsequently, the individual products of each gene were compared with the amplification product of ACT (as a normalizing gene), which was given a value (30 %). This served as a reference to estimate the hybridization of each gene in response to its band intensity on the agarose gel. For electrophoresis, 0.5 µg of product per lane was used and developed on 1X TAE agarose gels in the presence of ethidium bromide and exposed to UV light.

RESULTS

Extraction and purification of total RNA from cocoa roots and leaves: The method employed in the extraction of total RNA from roots was found to be of sufficient purity and yield for complementary DNA synthesis. Electrophoresis assays on agarose gels showed clear RNA bands, without traces of degradation and contamination by genomic DNA and proteins. The RNA obtained was subjected to quantification assays by spectrophotometry, taking into account the absorbance ratios. The A260/A280 ratio showed the absence of contaminating proteins in the extraction products, which ranged from 1.8-2. On the other hand, the 260/230 ratio showed a low level of contamination by polysaccharides, carbohydrates and other derivatives, which demonstrates that the extraction method used was efficient in the extraction and purification of total RNA, being of sufficient purity for the synthesis of complementary DNA and PCR amplification assays (Fig. 1).

Gene amplification by PCR: Agarose gel electrophoresis shows the PCR amplification products of the ACT, ODC, DEH, and ABS genes, using 50 ng of cDNA as a template in the PCR reaction. The size of the amplicons corresponds to the theoretical size expected for each gene (Table 1), except for the ABA gene which showed no amplification product in both rootstocks and grafted leaves (Fig. 2).

Hybridization profile of ODC, ABA, DEH, and ABS genes to actin (ACT): To construct the hybridization profile of each gene, the Actin band (ACT) was compared with the band intensity (of 200 bp) of the molecular weight marker TrackIt™ 100 bp DNA Ladder, Invitrogen (USA). After comparison, ACT was given a reference value of 20 %, which served as a reference to construct the hybridization profile for each gene. Thus, when comparing the hybridization profiles of the ODC, ABA, DEH, and ABS genes concerning actin (ACT), the ABS gene showed greater hybridization according to the intensity of the band in the gel, being 5 times (100 %) more intense than the normalizing ACT gene. On the other hand, the ODC gene was 2 times (40 %) more intense, while DEH showed hybridization below ACT, with 10 %. ABA did not show hybridization with root DNA in cocoa patterns (photo A). Meanwhile, when comparing the hybridization of genes in leaves from cocoa grafts, ABS and ODC reached hybridization levels above the normalizing gene (ACT), with 80 % and 25 %, respectively, while DEH showed hybridization below

ACT, with 5%. In this case, ABA also did not show hybridization with DNA from leaves derived from cocoa grafts (photo B). This shows that DEH and ABA genes hybridize at a very low level (not visible for ABA) in the experimental conditions studied for both rootstocks and grafts (Fig. 3).

Table 1. Oligonucleotides used

Oligonucleotide	Melting Temperature ºC	Sequence (5'-3')	Expected ampliconsize	NCBI Accession
*Tc*ABA_FWD	57.9	CAACTACCTGCTAGGCAATAG	843 pb	XM_018120132.1
*Tc*ABA_REV	56.7	CATCAGGGAACCTCAATGC		
TcODC_FWD	56.5	TCTCATCTTGCTGTTAAAAGCC	1340 pb	EF122792.1
TcODC_REV	54.5	GTCAAACACGAAACCATGC		
*Tc*ABS_FWD	55.3	AAGCATACTCAACCAACCAC	510 pb	XM_007019478.2
*Tc*ABS_REV	54.5	ACACAACACACGGAATGAC		
*Tc*ACT_FWD	61.3	ACCTCACTGATGCCCTGATGAAGAT	200 pb	XM_007026973.2
*Tc*ACT_REV	61.3	GATGGTGATCACCTGACCATCAGGC		
*Tc*Dehy_FWD	57.3	GTTGCGCGTAGTGAATGATG	1018 pb	XM_007017903.2
TcDehy_REV	55.9	CAATCAAAGCAGGAGGAAAGA		

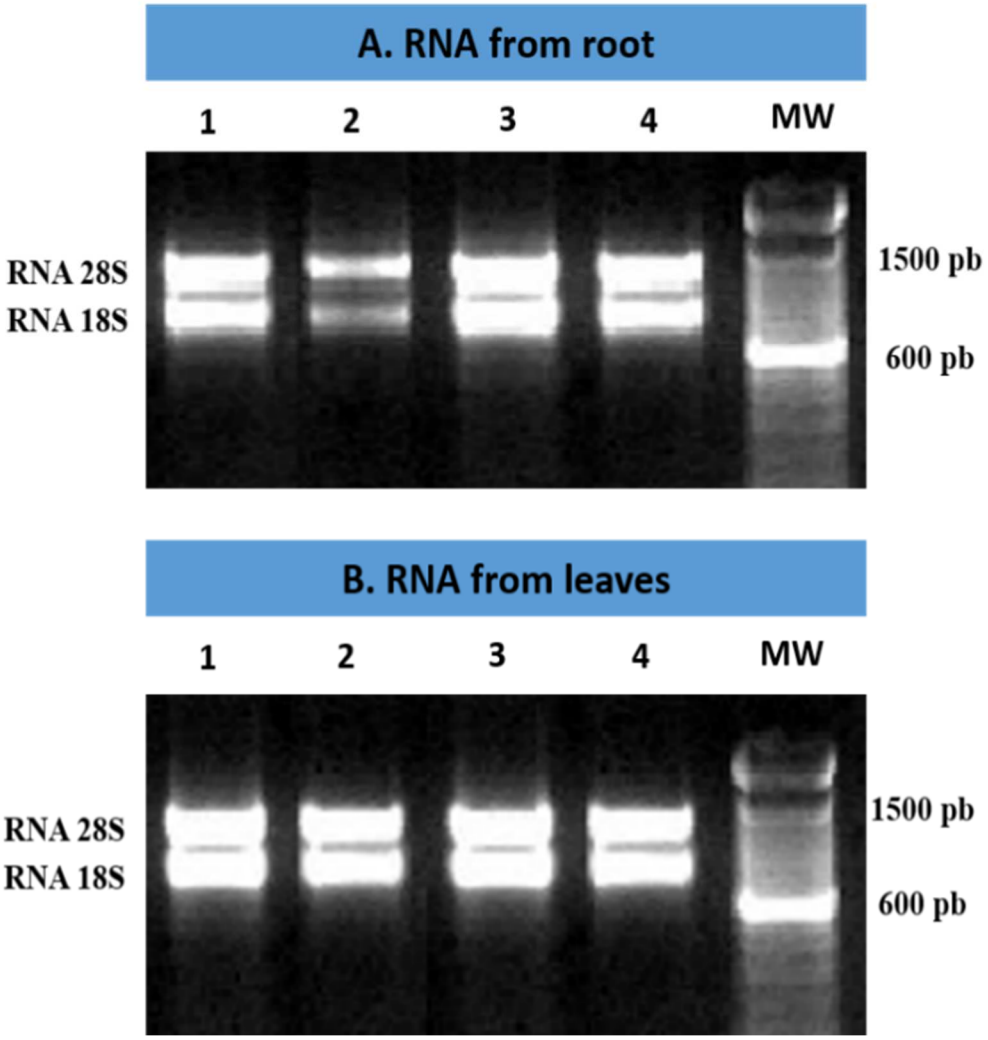

Figure 1. Electrophoresis of total RNA from roots (A) and leaves (B) of *T. cacao* in 1 % (w/v) agarose gels. *Photo A*, RNA from roots of rootstocks: BMI67 (lane 1); CCN51 (lane 2); EET400 (lane 3); EET399; (lane 4). *Photo B*, RNA from leaves of the graft: T800 (lane 1); T801 (lane 2); T12 (lane 4); T14 (lane 4). MW, Molecular weight marker (TrackIt™ 100 bp DNA Ladder, Invitrogen, USA).

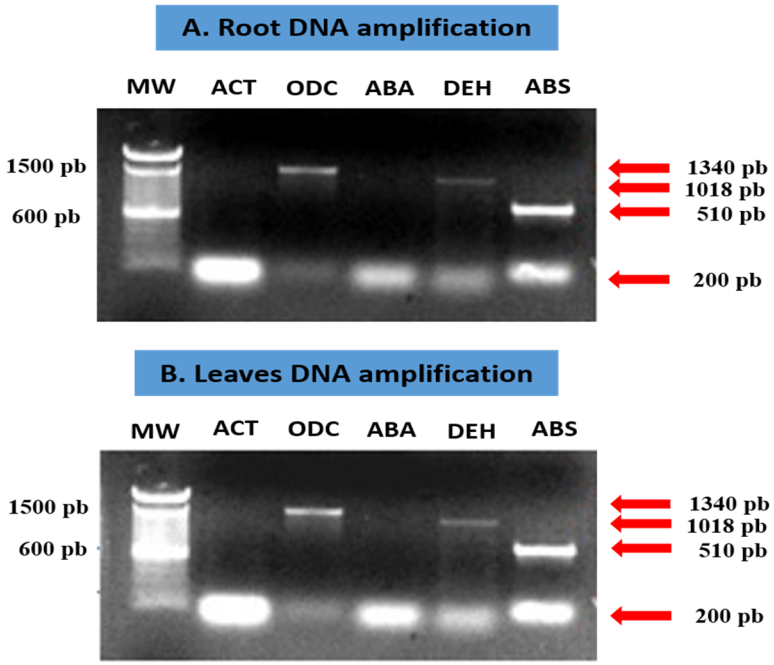

Figure 2. DNA amplification product of ACT, ODC, ABA. DEH and ABS genes in roots and leaves of *T. cacao*. on 1 % (w/v) agarose gels. *Photo A*, DNA amplification of roots of rootstocks: BMI67 (lane 1); CCN51 (lane 2); EET400 (lane 3); EET399; (lane 4). *Photo B*, DNA of grafted leaves: T800 (lane 1); T801 (lane 2); T12 (lane 4); T24 (lane 4). MW, Molecular weight marker (TrackIt™ 100 bp DNA Ladder, Invitrogen, USA).

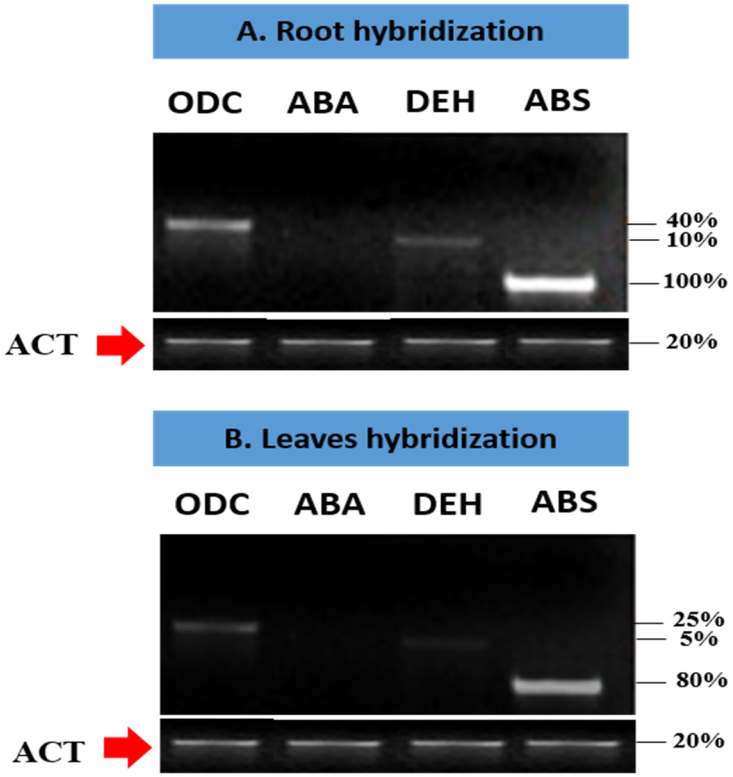

Figure 3. Hybridization profile of ODC, ABA, DEH, and ABS genes concerning ACT in rootstocks (IMC6, CCN51, EET400, EET399) of cocoa, respectively (*Photo A*) and in leaves of grafts (T800, T801, T12, T24), respectively (Photo B). MW, Molecular weight marker (TrackIt™ 100 bp DNA Ladder, Invitrogen, USA).

DISCUSSION

Drought is one of the most limiting problems in agricultural production, considering that climate change affects the frequency of precipitation and temperatures, thus reflecting water scarcity due to the lack of rainfall over extended periods (Pace *et al*., 2013; Jaimez *et al*. 2021). Leaf water potential, net photosynthesis, and cocoa seedling growth are physiological variables that are particularly sensitive to water deficit. Knowledge of the expression and genetic profiles in response to water deficit constitutes an advance in breeding programs.

For molecular analysis, the extraction of genetic material is a technique that guarantees the amplification and sequencing procedure. The purity and yield achieved in the RNA extraction protocol were similar to those reported by De Wever *et al*. (2020). They suggest that A260/230 purities are not influenced by how the RNA is separated from additional contaminants (silica gel column filters or pelleting), but by how the sample is homogenized allowing an optimal purity level between 1.8-2. Taking into account that cocoa has the presence of polyphenols, which makes the extraction of genetic material difficult, the purity and quality obtained corroborates what was expressed by (Martínez-López *et al*., 2013) who mentioned that some protocols comply with the characteristics of yield and quality, which can be used in the application of more advanced molecular techniques.

As reported in this study, the ABA gene hybridizes at a very low level in the experimental conditions studied in grafts. This is in agreement with Santos *et al*. (2014) who stated that ABA degradation accumulates and reduces its synthesis when genes are repressed during long-term treatment, as shown in tolerant cocoa cultivars. Another explanation for the decrease in ABA concentration may be the conversion of ABA to ABA-glycosyl ester, as recently reported for *Thellungiellasalsuginea* subjected to salt stress (Prerostova*et al*., 2017; Phillips-Mora and Wilkinson, 2007).

Concerning the above, plants have response mechanisms to different types of stress, biotic or abiotic, that contribute to their survival in adverse conditions. Some species show greater diversity in terms of favorable or unfavorable environmental responses and therefore, a higher survival rate compared to other species. In this sense, the study of wild species with these characteristics is important for the sustainability of food production.

Once the amplification products were obtained, the intensities of the DNA bands of each gene were compared with the bands of the reference gene (Actin). This was done to measure gene hybridization and create a profile for each plant under water deficit stress from leaves and roots of cocoa rootstocks and grafts. In many studies, the actin gene has been used as a reference for internal control in gene expression (Dheda *et al*., 2005).

This research is fundamental since it allows us to know the nucleotide diversity of the genes coding for water stress, since they may be related to adaptability and greater possibility of response to environmental stress. It also contributes to the understanding of the responses of these plants to water stress so that they can be used in crop improvement programs for *T. cacao*. Modern molecular selection techniques including marker-assisted selection (MAS) and quantitative trait loci (QTL) mapping are currently being employed in cocoa breeding programs (Lanaud *et al*., 2009; Pugh *et al*., 2004; Schnell *et al*., 2005). These can also be applied to cocoa rootstocks and grafts, to investigate the identification of water-deficit tolerant species.

The molecular responses to water deficit evaluated in this work allow the development of new molecular tools for mass cloning systems of *T. cacao* plants that involve the expression of these genes and that are perfectly adapted to water deficit conditions. Therefore, these plants should be replicated as a basis for genetic improvement studies and to promote agricultural production with tolerance characteristics of Ecuadorian cocoa to water stress.

Conclusions: The strategy employed in this work allowed the identification and characterization of four genes potentially involved in *T. cacao* responses to water deficit. Of these four genes, the ABS gene hybridized 100 % with DNA from rootstock EET399 in roots and 80 % with DNA from graft T24 in leaves. The OCD gene hybridized 80 % with DNA from rootstock IMC 67 and 25 % with the T800 graft in leaves. The DEH gene hybridized below the normalizing gene (ACT) in both rootstock EET400 (10 %) in root DNA and graft T12 (5 %) in leaves. Finally, the ABA gene did not hybridize with the DNA of the CCN51 rootstock or with the graft (T801) in both roots and leaves, respectively. This research is key to understand some molecular mechanisms of *T. cacao* in response to water deficit and can be considered as a viable strategy to reduce crop agronomic losses under the current environmental scenario.

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Conflicts of Interest: The authors declare that there are not conflicts of interest.

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