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Modulation of the transcriptional activity of the AP2/ERF family (DREB genes) in orange (*Citrus sinensis*) leaves subjected to drought stress

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Ethylene response factor (ERF) play important role in the development and expression of genes that regulate plant response to biotic and abiotic stresses. In this study, six *Citrus sinensis* genes belonging to AP2/ERF family, dehydration-responsive element-binding protein (DREB) genes, (*CitsERF01* to *CitsERF06)* were identified and five of them had their expression pattern evaluated in leaves of 18 month-seedling orange (*C. sinensis*) subjected to increasing intervals of drought stress. Two gene transcription patterns were identified. In the first pattern, transcription of *CitsERF01* and *CitsERF03* genes was delayed for many days and did not occur until the 12th day. In the second pattern, transcription of *CitsERF04, CitsERF05* and *CitsERF06* was immediate, and their relative value increased steadily, also reaching its peak at the 12th day. However, in both cases, transcription of *CitsERF* genes was down regulated with water recovery. These patterns suggest that *CitsERFs* genes may be involved in regulation mechanisms of drought response of *C. sinensis* and are controlled by factors acting in cascade. The data from this study will help understand the genetic mechanisms of drought tolerance, which could contribute to breeding programs of orange.

Key words: Gene expression, transcript factor; dehydration-responsive element-binding protein genes, semi-quantitative analysis RT-PCR.

INTRODUCTION

During their life cycle, plants are exposed to different adverse environmental events that affect their development and growth (Lee et al., 1999). To survive, the plants respond and adapt to these conditions with an array of biochemical and physiological changes. Many adaptation processes are regulated by stress responsive gene expression. Transcription factors play central roles in gene expression by regulating the expression of downstream genes, and transcription factors have important functions in the transcriptional regulation of a variety of biological

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Abbreviations: ERF, Ethylene response factor; DREB, dehydration-responsive element-binding protein; ORF, open reading frame.

processes, including various responses to the environment (Riechmann, 2000; Nakano et al., 2006; Chen et al., 2012). Among the different transcript factors, the ones from the AP2/ERF family play important role in the transcription regulation for increasing tolerance to drought, salinity, low and high temperatures, and plant diseases (Dubouzet et al., 2003; Zhuang et al., 2011). The ethylene response factor (ERF) family belongs to the AP2/ERF superfamily which is defined by the presence of preserved AP2/ERF domain consisting of a sequence of 60 to 70 amino acids involved in DNA binding (Jofuku et al., 1994; Riechmann et al., 2000; Hu and Liu, 2011). Proteins of ERF family have only one AP2 domain, and are classified into two subfamilies, ERF and DREB (dehydration-responsive element-binding protein) (Jofuku et al., 1994; Sakuma et al., 2002). The members of ERF family bind to the cis-regulatory element called GCC-*box*, in the gene-promoting regions related to pathogenesis and regulate the expression in response to dehydration and low temperature (Sharma et al., 2010). The superexpression of DREB genes in Arabidopsis activate the expression of many genes related to abiotic stress, improving tolerance to drought, salt and cold (Liu et al., 1998). Dobouzet et al. (2003) verified that the superexpression of *OsDREB1A* in transgenic Arabidopsis induced the expression of *DREB1A* genes, resulting in plants with greater tolerance to drought, salinity and frosting. Likewise, Bouaziz et al. (2012) revealed that the superexpression of *StDREB2* in transgenic potato resulted in greater tolerance to salt stress.

Orange fruit (*Citrus sinensis*) play important role in the economy of many countries around the world, but mainly in Brazil, USA and China (FAO, 2012). Adversely, the productivity and good quality of orange have been negatively affected by abiotic stresses like drought and salinity (Ben-Hayyim and Moore, 2007). Due to the importance of abiotic and biotic stress responses, AP2/ERF transcription factors represent interesting gene pools to be investigated for breeding and genetic engineering purposes (Xu et al., 2011). In this way, considering the importance of the subfamily DREB in citrus in relation to response and tolerance to abiotic stress, this study aimed to characterize the transcriptional modulation of five *CitsE.* The results of this study could be used to analyze the molecular mechanism underlying the stress tolerance of orange.

MATERIALS AND METHODS

Identification of AP2/ERF genes in *C. sinensis* genome

Protein sequences of DREB subfamilies belonging to group I, identified in *Arabidopsis thaliana* from TAIR database (http://www.arabidopsis.org/) were utilized for research in the Citrus Genome Database of *C. sinensis*, (http://www.citrusgenomedb.org/species/sinensis) using BlastP software (Altschul et al., 1997). The sequences were compared to other sequences stored in the GenBank database, utilizing BlastP and BlastX (National Center for Biotechnology Information - NCBI - http://www.ncbi.nlm.nih.gov), to confirm their identity. The deduced sequences of amino acids were obtained through Open Reading Frame Finder (ORF Finder - NCBI - http://www.ncbi.nlm.nih.gov/gorf/gorf.html) software. The initially collected sequences that presented incomplete AP2 domain or whose ORFs were incorrect were excluded from the analysis. The protein sequences containing AP2 domain were aligned utilizing ClustalW algorithm, version 2.0 (Larkin et al., 2007) and redundant inputs were removed.

Characteristics of predicted AP2/ERF proteins in citrus

The basic physical and chemical characteristics of citrus AP2/ERF proteins were calculated using the online ProtParam tool (http://web.expasy.org/protparam), including the number of amino acids, molecular weight and theoretical isoelectric point (pI). Analysis of the 3-D structure was completed on the online server Phyre v.2 (Kelley and Sternberg, 2009).

Phylogenetic analysis

The phylogenetic analysis was done by aligning AP2 domains of *C. sinensis* using ClustalW algorithm, version 2.0 (Larkin et al., 2007). Searches for other similar proteins were done with BlastP software (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was built by the neighbor-joining (NJ) method using the pair-wise deletion option with the help of MEGA software, version 5.10 (Tamura et al., 2011). To test the reliability of the analysis, 1,000 bootstrap replicas were utilized.

Plant material and stress treatment

In this study, we measured the transcription of five out of the six *CitsERFs* genes in leaves of orange (Pêra variety) subjected to increasing intervals of drought stress. In September 2012, 30 vigorous 18-month-old orange seedlings were brought from a nursery field to the greenhouse and received water at soil field capacity regularly for 15 days until the treatments started. The greenhouse was set up to maintain air temperatures of 25±5°C, relative air humidity of 80±10% and the light: dark periods of 16: 08 h. After the period of acclimation, we set up the 'control' pool treatment by harvesting all young mature leaves from three seedlings. Following that, the drought treatments consisted of not watering the seedlings for three, six and twelve days, consecutively. At each interval, we similarly harvested all young mature leaves from another group of seedlings. After 12 days of drought, three seedlings received water as in the start of the experiment for another three days and were harvested as the 'recovery' treatment. Each biological replicate consisted of a pool of leaves from three different orange seedlings. Harvested material was immediately immersed in liquid N and stored in freezer at -80°C.

RNA extraction and cDNA synthesis

Total RNA extraction of *C. sinensis* leaves was done for the drought stress experiment. Total RNA was extracted by utilizing SV Total RNA Isolation System commercial kit (Promega, Madison, USA) following the manufacturer's instructions. RNA integrity was analyzed in electrophoresis in agarose gel 1.2%. The first cDNA tape was synthesized utilizing 4 µL of total purified RNA and GoScript™ Reverse Transcription System commercial kit (Promega, Madison, USA) and the oligo primers $(dT)_{15}$ following the

Table 1. Sequence of primers utilized for semi-quantitative analysis by RT-PCR of *C. sinensis* genes.

Genes	Forward primers 5'-3'	Reverse primers 5'-3'	*T*m °C	Nº of cycles
ACTB	CCAATTCTCTCTTGAACCTGTCCTT	GAAGACCGTCAAGAGTAGTCAGT	56	32
CitERF01	TGACAGCTCTAACAATCTTCTC	GGTTTCATAGGACAAGGAGG	56	30
CitERF03	GCCAAGATTCAAGCAATATGTC	CTCCACTTTGTCACTCTCAG	56	30
CitERF04	TACTCTCCAACCACTTCTTCAG	TGAGATGATTTAGCCCAAGAG	56	30
CitERF05	TTACCAGTCTTACCCACCTC	TCATCGACAACCCATTAGCA	56	30
CitERF06	GAAAGAGAAGCAAAGTAACTCC	CTGACCCATCACTCAAACTG	56	30

manufacturer's instructions.

Semi-quantitative analysis by RT-PCR

The sequences of primers utilized for semi-quantitative analysis RT-PCR of *CitsERFs* genes and the *ACTB* gene (Yan et al., 2012) used as control, are given in Table 1. The reactions of RT-PCR considered a final volume of 15 µL, containing: 1.5 µL of 10x PCR buffer, 0.8 µL de $MgCl_2$ (50 mM), 0.5 µL of dNTP's (10 mM), 0,5 µL of each primer (10 mM), 0.4 µL of *Taq DN* thermocycler, model AG *A polymerase* (5U), 1 µl of diluted cDNA 1:10. The reactions occurred in a 22331 (Eppendorf, Hamburg, Germany), under the following conditions: 1 initial cycle at 94°C for 5 min and 30 to 32 cycles of 94°C for 30 s for denaturation, 56°C for 40 s for annealing, 72°C for 30 s for extension and 1 final cycle at 72ºC for 10 mi. The amplified PCR product was submitted to electrophoresis in agarose gel 1.2% stained with ethidium bromide. The gel images were captured utilizing a photo documenting systems, L-PIX - Molecular Imaging (Loccus Biotecnologia, Cotia, Brazil) and analyzed densitometrically (Freschi et al., 2009). To quantify the band intensity, IMAGEJ 1.46 (http://rsbweb.nih.gov/ij/download.html) software was used.

Statistical analysis

The results were evaluated and submitted to variance analysis and the averages were compared by Tukey's test at 5% probability, utilizing statistical software, SISVAR (Ferreira, 1999).

RESULTS

Molecular characteristics of ERFs of *C. sinensis*

From the *in silico* analysis of the Genome Database of *C. sinensis,* six ERFs belonging to DREB subfamily of group I were identified and named *C. sinensis ethylene-responsive element binding factor* (*CitsERF01-06*). The complete cDNA sequence of *CitsERF01* (orange1.1g039976m), *CitsERF02* (orange1.1g015775m), *CitsERF03* (orange1.1g045091m), *CitsERF04* (orange1.1g018103m), *CitsERF05* (orange1.1g017798m) and *CitsERF06* (orange1.1g021983m) is 1401, 1203, 939, 2071, 1985 and 1985 bp and codify proteins of 438, 400, 312, 360, 365 and 304 amino acid residues, respectively. The estimated molecular weight is 48.79, 44.73, 34.52, 39.97, 39.97 and 33.64 kDa, and the isoelectric point of 5.48, 8.27, 8.33, 6.32, 7.62 and 8.48 for *CitsERF01* to *06,* respectively. The 3-D structures of predicted from group I to AP2/ERF family proteins in citrus were built through the on online server Phyre 2. As shown in Figure 1, each 3-D structure of the citrus AP2/ERF family protein contains one α-helix and three β-sheets. The group I of AP2/ERF family generally has similar 3-D structures, with slight differences in the length and amino acid composition of units that make up the tertiary structures. This is due to the number of amino acids in each β-sheet of each 3-D structure was different, thus creating slight variations in the length of β-sheets of each tertiary structure. Research done in NCBI by BLAST indicated that the six ERF proteins have great similarity with the AP2/ERF transcription factors of other perennial species. *CitsERF01*, *CitsERF02* and *CitsERF04* share 62, 53 and 62% of identity with the sequence of AP2/ERF EOX99382.1, EOY05285.1 and EOX92151.1 transcription factors, respectively in *Theobroma cacao*. *CitsERF03* and *CitsERF05* share 70 and 60% of identity with XP_002304554.1 and AFY98895.1 sequences in *Populus trichocarpa* and *Jatropha curcas,* respectively. *CitsERF06* shares 63% of identity with DREB1p (ADX97444.1) identity in *Hevea brasiliensis*. The predict-tion of amino acid sequences of *C. sinenis* in relation to the complete sequence is indicated in percentage of identity and similarity (Table 2). The identity ranged from 22.76% (*CitsERF01* and *CitsERF03*) to 100% (*CitsERF05* and *CitsERF06*) and presented the average of 35%. For similarity, the variation was 17.25% (*CitsERF01* and *CistERF02*) and 100% (*CitsERF05* and *CitsERF06*) and the average of 33% in all ERFs of the studied group.

The homology research by Blastp showed that the alignment of ERFs sequences of *C. sinensis* has a conserved region of DNA binding, AP2/ERF domain with 58 amino acids, which is characteristic of the family of ERF genes in plants. Moreover, the ERF domain of citrus proteins showed high sequence homology with other characterized species (Figure 2). DREB gene subfamily includes two main amino acid residues in AP2 domain, valine (V) and glutamic acid (E) in positions 14 and 19, respectively (Sakuma et al, 2002). All genes presented

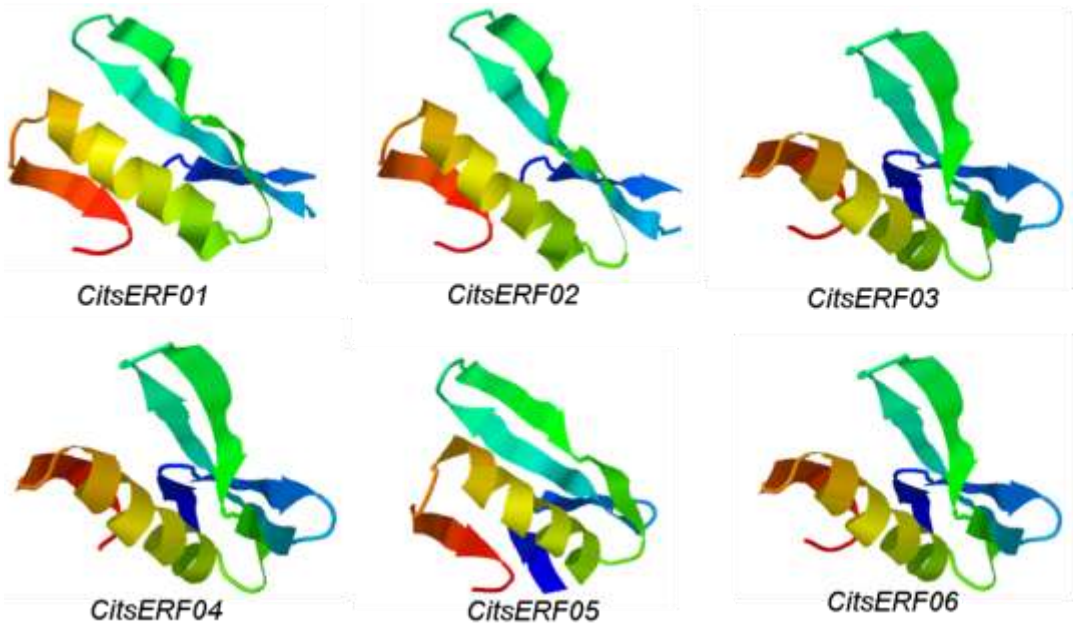

Figure 1. 3D structures of six AP2/ERF family proteins of group I in citrus. *CitsERF01* (orange1.1g039976m), *CitsERF02* (orange1.1g 015775m), *CitsERF03* (orange1.1g045091m), *CitsERF04* (orange1.1g018103m), *CitsERF05* (orange1.1g017798m) and *CitsERF06* (orange1.1g021983m); yellow-green = 1 alpha helix; green-blue = 3 beta sheets extended strand.

Table 2. Comparison among the predicted complete sequences of ERFs amino acids of *C. sinensis* (CitsERFs) cDNAs. The similarity values are over the diagonal whereas the identity values are under the diagonal.

	No. aa	*CitsERF01*	*CistERF02*	*CitsERF03*	*CitsERF04*	*CitsERF05*	*CitsERF06*
CitsERF01	438	-	17.25	22.76	21.11	24.66	26.64
CistERF02	400	22.82	-	24.04	23.89	25.21	25.66
CitsERF03	312	22.76	27.02	-	24.36	29.17	29.28
CitsERF04	360	24.29	29.94	29.53	-	48.33	51.97
CitsERF05	365	25.28	25.00	30.20	53.67	-	100.00
CitsERF06	304	27.30	28.57	30.30	55.89	100.00	-

No. aa: Number of amino acids

the preserved residue V14, and most genes presented leucine in position 19. In addition, alanine was found in position 37, and it was present in consensus in all protein sequences, including Arabidopsis, peach, grape, apple, rice and populus.

Phylogenetic analysis of CitsERF proteins

A phylogenetic analysis was done based on the sequence of ERF amino acids to determine the relation between citrus ERF and other characterized plant species (Figure 3). The analysis showed a distribution of protein sequences in four groups belonging to DREB subfamily and six *CitsERF* proteins were classified as members of group I; the other characterized proteins were divided into groups I, II, III and IV according to the classification proposed by Nakano et al. (2006).

CitsERF expression in response to stress

To determine the response of *CitsERF* subjected to drought stress, the corresponding mRNAs levels were quantified. Out of six *CitsERFs* identified in group I, five had their transcriptional activity evaluated. When subjected to stress, *CitsERF01* and *CitsERF03* (Figure 4) presented a similar response pattern, where it was observed that the expression was constant between the control (day 0), three and six days after the beginning of the stress, and there was a significant increase in transcripts at 12 days of stress, reducing to basal levels

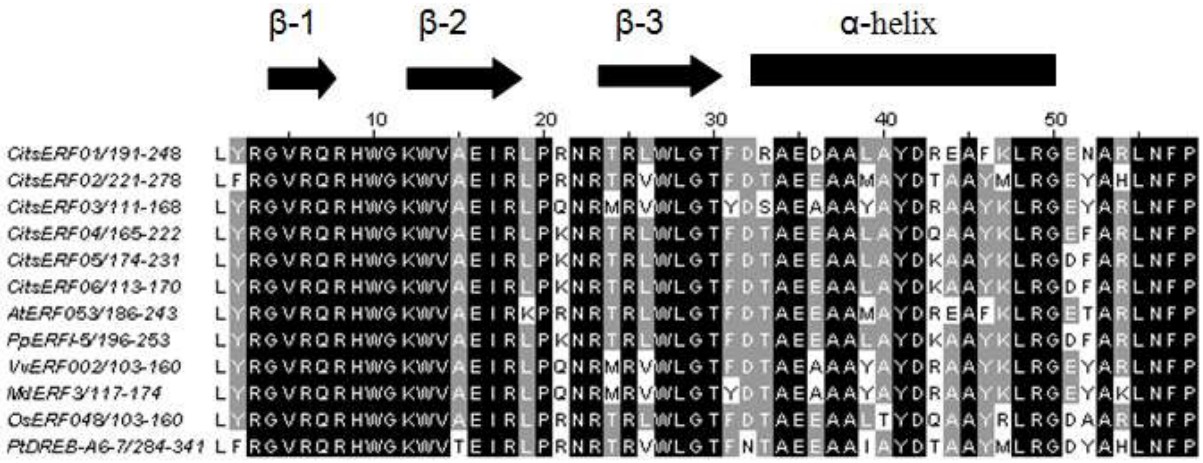

Figure 2. Alignments of AP2/ERF domains from *C. sinensis* proteins with other plant proteins, *A. thaliana* AtERF053 (AT2G20880), *P. persica* PpERFI-5 (ppa007193m), *V. vinifera* VvERF002 (GSVIVP00002438001) and *M. domestica* MdERF3 (MDP0000292965), *O. sativa* OsERF048 (Os08g31580) and *P. trichocarpa* PtDREB-A6-7 (eugene3.00012264). The black and gray columns indicate amino acid residues that are identical and preserved, respectively. The black bar and the arrows represent the α-helix and β-sheet regions, respectively, within AP2/ERF domain (Allen et al., 1998).

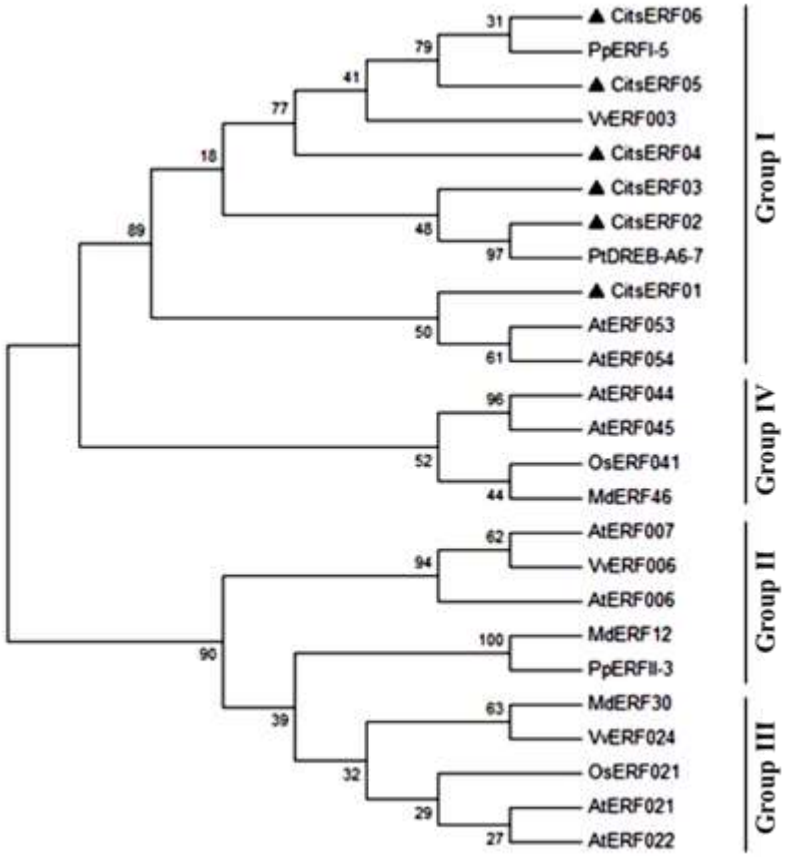

Figure 3. Phylogenetic analysis of DREB subfamily of *C. sinensis* and other plant organisms. The citrus proteins are marked with ▲. The names of the other proteins and their respective access numbers are *Arabidopsis thaliana* AtERF006 (AT1G46768), AtERF007 (AT4G06746), AtERF021 (AT1G71450), AtERF022 (AT1G33760), AtERF044 (AT3G11020), AtERF045 (AT5G05410), AtERF053 (AT2G20880), AtERF054 (AT4G28140); *Vitis vinifera* VvERF003 (GSVIVP00010923001), VvERF006 (GSVIVP00014863001) VvERF024 (GSVIVP00016137001); *Oryza sativa* OsERF021 (Os02g35240), OsERF041 (Os03g07830); *Malus x domestica* MdERF12 (MDP0000923690), MdERF30 (MDP0000652413), MdERF46 (MDP0000139446); *Populus trichocarpa* PtDREB-A6-7 (eugene3.00012264) and *P. persica* PpERFI-5 (ppa007193m), PpERFII-3 (ppa010649m).

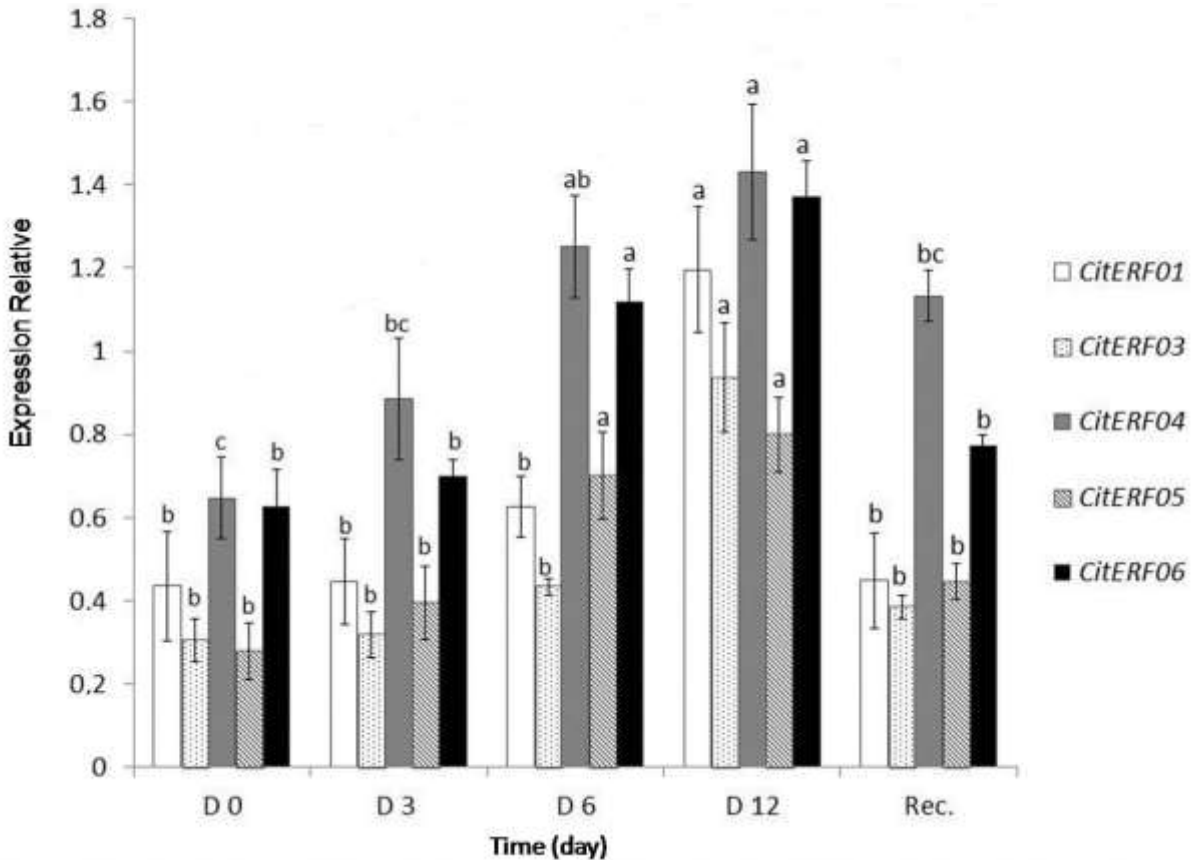

Figure 4. Changes during treatment in mRNA levels of *CitsERF01*, *CitsERF03*, *CitsERF04, CitsERF05* and *CitsERF06* in response to drought stress treatment (D0 – control, D3 – day 3, D6 – day 6, D12 – day12 and Rec – recovery (72 h of irrigation after the plants reached 12 days of stress). Transcript level of each sample was quantified by densitometry and normalized according to its corresponding *ACTB* density value (*CitsERF/ACTB*). The values represent the averages ± standard deviation of three biological replications. Averages followed by the same low case letters do not differ among the treatments by Tukey's test at 5%.

at recovery. *CitsERF04* (Figure 4) had a gradual increase of transcripts starting at control, 3 and 6 days after the beginning of the stress and reaching maximum activity 12 days after the beginning of the treatment and decreasing at recovery, but still at a higher level than the control. For *CitsERF05* and *CitsERF06* (Figure 4), it was observed that, between the control day and three days of stress, there was a small increase of expression and, after six days of stress, there was a greater transcriptional activity, reaching the maximum accumulation of transcripts at 12 days without water addition and reducing at recovery, after the stress. Based on the transcriptional profile of ERF genes subjected to drought stress, two expression standards were identified (Figure 5). In the first standard, *CitsERF01* and *03* exhibited expression at basal levels in control and at three and six days, whereas at 12 days, the maximum transcriptional activity was observed. In the second standard, it was verified that *Cits04, 05* and *06* showed a very similar expression standard among themselves at the beginning of the stress (D0, D3 and D6), reaching the maximum 12 days after the beginning of the stress and decreasing at recovery.

DISCUSSION

Transcription factors (TF) are regulators that control biological processes and have been considered as an important tool in the complex metabolic ways in plants (Grotewolda, 2008). TFs of ERF family are involved in the response to biotic and abiotic stresses and have a highly-preserved element that includes an AP2 domain which is essential for the plant survival (Riechmann and Meyerowitz, 1998; Sharma et al., 2010). In this study, the group I of DREB subfamily in citrus was analyzed and the identity among ERFs was high, varying from 22.76 to 100%, and all six *CitsERFs* presented a highly-preserved element, AP2/ERF domain. The presence of this domain is responsible for the DNA-binding activity (Fujimoto et al., 2000). AP2/ERF domain consists of 58 amino acids and has three anti-parallel β-*sheets* and one α-*helix*. This structure has a fundamental role in the recognition of the

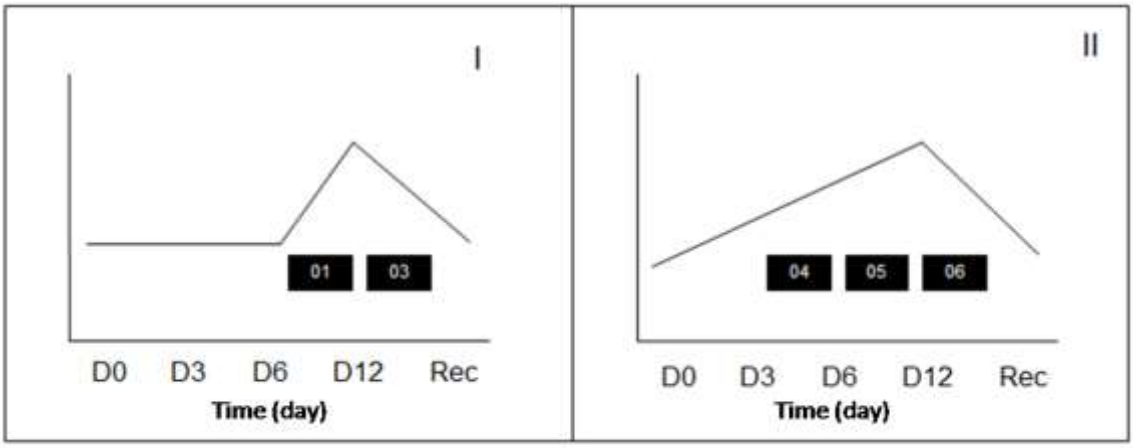

Figure 5. Two patterns expression during drought stress. Axis x represents: D0-control, D3- day 3, D6- day 6, D12- day 12 and Rec – recovery (72 h of irrigation after the plants reached 12 days of stress).

specific binding to cis-element (Allen et al., 1998; Sakuma et al., 2002). This observation is generally consistent with that in *Arabidopsis*, whereby the AP2/ERF domain is reported to contain an N-terminal, which is a three-stranded β-sheet that recognizes a target sequence as well as a C-terminal α-helix (Allen et al., 1998; Zhang et al., 2012). Due to the preserved residues, it was possible to identify that in DREB subfamily, all proteins had valine residue in position 14, but in position 19, most of them presented leucine. In previous studies, it is suggested that valine 14 and glutamic acid 19 in the AP2/ERF domain are essential to the specific binding to the element that is responsive to dehydration (Cao et al., 2001; Sakuma et al., 2002). However, this difference found in citrus was also found in other plants including Arabidopsis (Nakano et al., 2006), cotton (Champion et al., 2009), peach (Zhang et al., 2012) and apple (Zhuang et al., 2011). These observations suggest that the function of valine 14 is probably more important than the amino acid in position 19 for the DNA-binding activity (Wang et al., 2011). Alanine in position 37 of AP2/ERF domain found in citrus can be essential to the binding stability of ERF domain or the binding with DRE element or GCC box (Liu et al., 2006).

Overall, the genes of CBF/DREB subfamily have an important role in plant tolerance to abiotic stress, recognizing the dehydration responsive element (DRE), with TACCGACAT sequence, known as a cis-acting element that responds to cold or osmotic stress. DREB domain binds to the DRE element and regulates expression in response to dehydration and low temperature (Shinozaki and Shinozaki, 1994; Sakuma et al., 2002). In a previous study, Shinozaki and Shinozaki (1994) verified that the dehydration responsive element (DRE) is involved in the response of rd29A promoter in Arabidopsis and transgenic tobacco under drought or salinity conditions. The activity of most genes of ERF family is related to the increase of plant tolerance to biotic and abiotic stresses (Park et al., 2001; Guo et al., 2004; Zhang et al., 2009). Based on the phylogenetic analysis and the response expression to abiotic stress, most *CitsERFs* have a possible role as transcription activators that can be part of mechanisms of tolerance increase to abiotic stress in *C. sinensis*. Five analyzed ERFs were differently regulated at mRNA levels. These results suggest that all *CitsERFs* are up-regulated during drought stress, where the maximum level of the transcriptional activity was accumulated on the most severe day of stress, decreasing at recovery. High expression levels of *CitERF* under drought conditions were also observed in citrus plants, showing that it can be involved in the tolerance mechanisms to drought (Yan et al., 2011). High levels of DREBs *ZmDBF1* and *GhDBP2* were found in corn and cotton, respectively, when the plants were subjected to drought (Kizis and Pages, 2002; Huang et al., 2008). The authors showed that *ZmDBF1* as well as *GhDBP2* can be involved in the regulation of some late embryogenesis abundant (LEA) genes. These proteins accumulate during the advanced stages of embryogenesis and also in tissues exposed to stress like dehydration, osmotic stress and low temperature, and have an important role in protein stabilization and membrane structure during cell dehydration (Hong-Bo et al., 2005). Likewise, Bouaziz et al. (2012) attributed tolerance to drought and salinity of potato *StDREB1* to the expression of StCDPK4 and *StCDPK5* genes induced by stress that activates Ca^{2+} channels of the plasmatic membrane and *P5CS* gene which is responsible for the biosynthesis of proline, an important osmoprotector. The data found in this study are in accordance with other reports found in literature that revealed that the members of ERF family have important roles in response to abiotic stresses through the regulation of numerous stress induced genes (Agarwal et al., 2006). However, future studies must be carried out to identify the possible genes and the multiple ways of regulated transduction by

CitsERFs found in this study.

CitsERF genes of citrus responded differently to stress. It is noteworthy that exposure of orange seedlings to growing periods of drought resulted in two patterns of *CitsERF* gene transcription (Figure 5). In the first pattern, transcription of *CitsERF01* and *CitsERF03* genes was delayed for many days and did not occur until the 12th day. In the second pattern, transcription of *CitsERF04, CitsERF05* and *CitsERF06* was immediate, and their relative value increased steadily, also reaching its peak at the 12th day. However, in both cases, transcription of *CitsERF* genes was down regulated with water recovery. In fact, the two different patterns could be explained by the fact that the regulation mechanisms and gene expression due to environmental stress are many times controlled by factors acting in cascade (Riechmann, 2000). Earlier responses are possibly related to genes that encode signaling or regulatory components such as protein kinases and transcript factors. Later responses are often related to genes that encode "effector proteins" such as enzymes in the metabolism (Du and Chen, 2000). Plants may regulate their response to stress with the transcription of genes at early stage to prevent damage, and at later stage to limiting damage-processes. The early response is quickly induced but it is often transient. This induction does not need new protein synthesis because all signaling components are already in place (Zhu, 2002). On the other hand, the late response genes are activated more slowly by stress and their expression is many times kept (Cheong et al., 2002). Thus, probably the two types of responses found in this work are involved in plant tolerance to drought. In several cases, the protein products of early response function to regulate the expression of late response, suggesting a cascade of gene regulation (Cheong et al., 2002). Nevertheless, it is certain that further studies with genetic and biochemical approaches are required to test a model for the *CitsERF* genes pathways.

Conclusion

In this study, it was possible to identify that the genes of DREB subfamily in citrus have a crucial role in the development and regulation of this plant as well as in the responses to environmental stress. Thus, the data obtained in this study provide resources to select the genes for future functional analyses of ERF family in *C. sinensis*, which will help understand the genetic determiners of tolerance to abiotic stresses. This will be a fundamental step to genetic improvement programs of citrus to better the production and quality of fruits in limiting environmental situations.

Conflict of interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Agarwal P, Agarwal P, Reddy M, Sopory S (2006). Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep. 25:1263-1274.

Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M, Suzuki M (1998). A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. Embo J. 17: 5484-5496.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Ben-Hayyim G, Moore GA (2007). Recent advances in breeding citrus for drought and saline stress tolerance. In: Jenks MA, Hasegawa PM, Jain SM (Ed.): Advances in Molecular Breeding Toward Drought and Salt Tolerance Crops, pp. 627-642. Springer, Netherlands.

Bouaziz D, Pirrello J, Ben Amor H, Hammami A, Charfeddine M, Dhieb A, Bouzayen M, Gargouri-Bouzid R (2012). Ectopic expression of dehydration responsive element binding proteins (StDREB2) confers higher tolerance to salt stress in potato. Plant Physiol. Biochem. 60: 98-108.

Cao ZF, Li J, Chen F, Li YQ, Zhou HM, Liu Q (2001). Effect of two conserved amino acid residues on DREB1A function. Biochem. 66: 623-627.

Champion A, Hebrard E, Parra B, Bournaud C, Marmey P, Tranchant C, Nicole M (2009). Molecular diversity and gene expression of cotton ERF transcription factors reveal that group IXa members are responsive to jasmonate, ethylene and Xanthomonas. Mol. Plant Pathol. 10:471-485.

Chen N, Yang Q, Su M, Pan L, Chi X, Chen M, He Y, Yang Z, Wang T, Wang M, Yu S (2012). Cloning of six ERF family transcription factor genes from peanut and analysis of their expression during abiotic stress. Plant Mol. Biol. Rep. 30:1415-1425.

Cheong YH, Chang HS, Gupta R, Wang X, Zhu T, Luan S (2002). Transcriptional profiling reveals interactions between wounding, pathogen, abiotic stress, and hormonal response in Arabidopsis. Plant Physiol. 129: 661-677.

Du L, Chen Z (2000). Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in Arabidopsis. Plant J. 24: 837-847.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003). *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J. 33: 751-763.

FAO - Food and Agriculture Organization of the United Nations (2012). Available in: <http://www.fao.org/docrep/006/y5143e/y5143e12.htm> Retrieved on: 14 Nov. 2012.

Ferreira DF (1999). Sistema de análise de variância (Sisvar). Versão 4.6. Lavras: Universidade Federal de Lavras. (CD-ROM).

Freschi L, Nievola CC, Rodrigues MA, Domingues DS, van Sluys MA, Mercier H (2009). Thermoperiod affects the diurnal cycle of nitrate reductase expression and activity in pineapple plants by modulating the endogenous levels of cytokinins. Physiol. Plantarum, 137: 201-212.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression. Plant Cell, 12:393-404.

Grotewolda E (2008). Transcription factors for predictive plant metabolic

engineering: are we there yet? Curr. Opin. Biotech. 19:138-144.
Guo ZJ, Chen XJ, Wu XL, Ling JQ, Xu P (2004). Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. Plant Mol. Biol. 55: 607-618.
Hong-Bo S, Zong-Suo L, Ming-An S (2005). LEA proteins in higher plants: Structure, function, gene expression and regulation. Colloids and Surfaces B. 45:131-135.
Hu L, Liu S (2011). Genome-wide identification and phylogenetic analysis of the ERF gene family in cucumbers. Genet. Mol. Biol. 34: 624-633.
Huang B, Jin LG, Liu JY (2008). Identification and characterization of the novel gene GhDBP2 encoding a DRE-binding protein from cotton (*Gossypium hirsutum*). J. Plant Physiol. 165:214-223.
Jofuku KD, den Boer BG, van Montagu M, Okamuro JK (1994). Control of *Arabidopsis* flower and seed development by the homeotic gene APETALA2. Plant Cell, 6:1211-1225.
Kelley LA, Sternberg MJE (2009). Protein structure prediction on the web: a case study using the Phyre server. Nat. Protoc. 4: 363-371.
Kizis D, Pages M (2002). Maize DRE-binding proteins DBF1 and DBF2 are involved in rab17 regulation through the drought-responsive element in an ABA-dependent pathway. Plant J. 30: 679-689.
Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. Bioinformatics, 23: 2947-2948.
Lee H, Xiong L, Ishitani M, Stevenson B, Zhu JK (1999). Cold-regulated gene expression and freezing tolerance in an *Arabidopsis thaliana* mutant. Plant J. 17:301-308.
Liu Y, Zhao TJ, Liu JM, Liu WQ, Liu Q, Yan YB, Zhou HM (2006). The conserved Ala37 in the ERF/AP2 domain is essential for binding with the DRE element and the GCC box. FEBS Lett. 580: 1303-1308.
Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell, 10:1391-1406.
Nakano T, Suzuki K, Fujimura T, Shinshi H (2006). Genome wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol. 140: 411-432.
Park JM, Park CJ, Lee SB, Ham BK, Shin R, Paek KH (2001). Overexpression of the tobacco Tsi1 gene encoding an EREBP/ AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell, 13:1035-1046.
Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G (2000). Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Sci. 290: 2105-2110.
Riechmann JL, Meyerowitz EM (1998). The AP2/EREBP family of plant transcription factors. Biol. Chem. 379 (6): 633-646.
Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002). DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold inducible gene expression. Biochem. Biophys. Res. Commun. 290: 998-1009.
Sharma MK, Kumar R, Solanke AU, Sharma R, Tyagi AK, Sharma AK (2010). Identification, phylogeny, and transcript profiling of ERF family genes during development and abiotic stress treatments in tomato. Mol. Genet. Genomics 284: 455-475.
Shinozaki YK, Shinozaki K (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell, 6(2): 251-264.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). Mega 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731-2739.
Yan J, Yuan F, Long G, Qin L, Deng Z (2012). Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. Mol. Biol. Rep. 39:1831-1838.
Wang X, Chen X, Liu Y, Gao H, Wang Z, Sun G (2011). CkDREB gene in Caragana korshinskii is involved in the regulation of stress response to multiple abiotic stresses as an AP2/EREBP transcription factor. Mol. Biol. Rep. 38(4):2801-2811.
Xu ZS, Chen M, Li LC, Ma YZ (2011). Functions and application of the AP2/ERF transcription factor family in crop improvement. J. Integr. Plant Biol. 53:570-585.
Zhang G, Chen M, Li L, Xu Z, Chen X, Guo J, Ma Y (2009). Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. J. Exp. Bot. 60 (13): 3781-3796.
Zhang CH, Shangguan LF, Ma RJ, Sun X, Tao R, Guo L, Korir NK, Yu ML (2012). Genome-wide analysis of the AP2/ERF superfamily in peach (*Prunus persica*). Genet. Mol. Res. 11(4): 4789-4809.
Zhu JK (2002). Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53:247-273.
Zhuang J, Quan-Hong Y, Xiong AS, Zhang J (2011). Isolation, phylogeny and expression patterns of AP2-Like genes in apple (Malus × domestica Borkh). Plant Mol. Biol. Rep. 29 (1): 209-216.