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TRANSCRIPTIONAL ANALYSIS OF SOYBEAN (*Glycine max*) GENES RELATED TO WATER DEFICIT

ANÁLISE TRANSCRICIONAL DE GENES RELACIONADOS AO DÉFICIT HÍDRICO EM SOJA (Glycine max)

ANÁLISIS TRANSCRIPCIONAL DE GENES RELACIONADOS CON EL DÉFICIT HÍDRICO EN SOJA (Glycine max)

Adriano Sérgio Bernardo Queiroz:
Mestre em Agroenergia Digital, Universidade Federal do Tocantins, Palmas, Tocantins, Brasil.
E-mail: adrianosbq@gmail.com. ORCID ID: https://orcid.org/0000-0002-1823-3916.

Antônia Clemilda Nunes:
Doutora e Servidora da Universidade Federal do Tocantins, Palmas, Tocantins, Brasil.
E-mail: clemilda@uft.edu.br. ORCID ID: https://orcid.org/0000-0002-7833-5623.

Matheus Martins Daúde:
Pós-Doutor do Laboratório de Análises Moleculares (LAM), Universidade Federal do Tocantins, e docente do ITPAC, Palmas, Tocantins, Brasil.
E-mail: matheusdaude@gmail.com. ORCID ID: https://orcid.org/0000-0001-6978-493X.

Horllys Gomes Barreto:
Docente do Programa de Pós-Graduação em Agroenergia Digital , Universidade Federal do Tocantins, Palmas, Tocantins, Brasil. E-mail: horllys@uft.edu.br. ORCID ID: https://orcid.org/0000-0001-7236-6296.

Solange Aparecida Ságio:
Docente do Programa de Pós-Graduação em Agroenergia Digital , Universidade Federal do Tocantins, Palmas, Tocantins, Brasil. E-mail: solangesagio@mail.uft.edu.br. ORCID ID: https://orcid.org/0000-0002-9329-4516.

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RESUMO

A cultura da soja vem ganhando destaque mundial, sendo amplamente difundida devido às suas variadas formas de utilização em diferentes segmentos. Fatores genéticos e ambientas são determinantes na qualidade dessa cultura. Uma característica relacionada ao fator ambiental que tem ganhado destaque é o déficit hídrico. Eventos moleculares são iniciados pela percepção do estresse hídrico, envolvendo uma complexa rede genética. Estudos nas últimas décadas já identificaram alguns genes relacionados ao estresse hídrico em plantas, sabe-se que a proteína de ligação a elementos responsivos à desidratação (DREB) desempenha um papel importante na resposta ao déficit hídrico. Diante disso este trabalho teve como objetivo analisar a expressão de genes relacionados ao déficit hídrico na cultura da soja. Para a análise da expressão gênica foram utilizadas folhas de soja (*Glycine max* (L.) Merrill), da cultivar M9144 RR, submetidas aos seguintes tratamentos: 25V25R – plantas submetidas ao déficit hídrico durante todo o ciclo, sendo irrigada com 25% da ETpc e 100V100R – plantas que não sofreram déficit hídrico, sendo irrigadas com 100% da ETpc durante todo o ciclo. A quantificação da expressão relativa dos genes GmDREB1 e GmDREB5 mostraram que o estresse hídrico sofrido pela cultivar foi maior no estádio reprodutivo.

Palavras-chave: Fatores de transcrição, estagios de desenvovimento, expressão gênica.

ABSTRACT

The soybean crop has been gaining worldwide prominence, being widely disseminated due to its various forms of use in different segments. Genetic and environmental factors are decisive in the quality of this culture. A feature related to the environmental factor that has gained prominence is the water deficit. Molecular events are initiated by the perception of water stress, involving a complex genetic network. Studies in recent decades have already identified some genes related to water stress in plants, it is known that the dehydration-responsive element binding protein (DREB) plays an important role in the response to water deficit. Therefore, this work aimed to analyze the expression of genes related to water deficit in soybean. For the analysis of gene expression, leaves of soybean (Glycine max (L.) Merrill), cultivar M9144 RR, submitted to the following treatments were used: 25V25R – plants subjected to water deficit throughout the cycle, being irrigated with 25% of ETpc and 100V100R – plants that did not experience water deficit, being irrigated with 100% of ETpc throughout the cycle. The quantification of the relative expression of the genes GmDREB1 and GmDREB5 showed that the water stress suffered by the cultivar was greater in the reproductive stage.

Keywords: *Transcription factors, development stages, gene expression.*

RESUMEN

El cultivo de la soja viene ganando protagonismo a nivel mundial, siendo ampliamente difundido debido a sus diversas formas de uso en diferentes segmentos. Los factores genéticos y ambientales son determinantes en la calidad de este cultivo. Una característica relacionada con el factor ambiental que ha ganado protagonismo es el déficit hídrico. Los eventos moleculares se inician por la percepción del estrés hídrico, lo que implica una red genética compleja. Estudios en las últimas décadas ya han identificado algunos genes relacionados con el estrés hídrico en las plantas, se sabe que la proteína de unión al elemento sensible a la deshidratación (DREB) juega un papel importante en la respuesta al déficit hídrico. Por lo tanto, este trabajo tuvo como objetivo analizar la expresión de genes relacionados con el déficit hídrico en soja. Hojas de soja (Glycine max (L.) Merrill), cultivar M9144 RR, fueron sometidas a los siguientes tratamientos para el análisis de expresión génica: 25V25R – plantas sometidas a déficit hídrico durante todo el ciclo, siendo regadas con 25% de ETpc y 100V100R – plantas que no experimentaron déficit hídrico, siendo regadas con el 100% de ETpc durante todo el ciclo. La cuantificación de la expresión relativa de los genes GmDREB1 y GmDREB5 mostró que el estrés hídrico que sufrió el cultivar fue mayor en la etapa reproductiva.

Descriptores: *Factores de transcripción, etapas de desarrollo, la expresion genica.*

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is an important crop for the world economy due to its diverse applicability in different sectors, such as in the human and animal feeding, biodiesel production, and raw material for industry (BATTISTI; SENTELHAS, 2019). The widespread use of soybean has made it the oilseed crop most cultivated in the world (LIU et al., 2020). Worldwide, the soybean estimated production for the year 2020/2021 is 362.8 million tons (USDA, 2020). Brazil, the world biggest producer of soybean, produced 120.3 million tons in the 2019/2020 harvest, with record yields from the Brazilian states of Mato Grosso, Paraná, Goiás, São Paulo, Tocantis, Maranhão, Rondônia, and Distrito Federal (CONAB, 2020). In the past few decades, soybean has consolidated as the main crop of the Brazilian agribusiness. Along this period, the grain led the expansion of the national agricultural frontier and started to be cultivated in various production systems (HIRAKURI et al., 2017). However, the implantation of this culture in new cultivation areas has required new technologies and adequate cultural management (PETTER et al., 2014)

Under natural conditions, plants are subjected to environmental variations through biotic and abiotic stresses. In relation to abiotic stresses, water deficit stands out, being the factor with the greatest impact on yield of several crops of economic importance, including soybean. Soybean plants can be influenced by environmental changes, affecting the chemical composition of the seeds, such as the grain protein and oil contents, which in turn determine the soybean quality. In this sense, water deficit is considered one of the factors with the greatest impact in soybean grain production and quality, leading to considerable decreases in yield, even in cultivars with excellent genetic potential (NUNES, 2015; MONTEIRO et al., 2017). Thus, plants use different mechanisms to protect themselves against drought, including modifications on stomatal aperture, osmotic adjustment, accumulation of osmoprotective molecules, and the activity of antioxidant enzymes (MARCOLINO-GOMES et al., 2015).

Water constitutes approximately 90 % of the weight of a soybean plant and acts in practically every physiological and biochemical of the plant. Water availability is most important in two soybean developmental stages: germination/seedling emergence and flowering/grain filling (FARIAS et al., 2007; VEIGA et al., 2010; MORANDO et al., 2014). The water requirements of the soybean crop increase according to the development of the plant, with its maximum demand during flowering and the onset of grain development. The lack of water at the beginning of the soybean cycle can cause strong reduction in the emission of new branches, potentially reducing the number of pods in the producing

nodes. However, if water is available after flowering, soybean plants can partially recover, emitting and fixing a higher number of flowers in the new nodes, reducing the formation of new leaves as well, supporting grain filling (MUNDSTOCK; THOMAS, 2005).

The agronomic characteristics in a crop determine the plant's phenotype, which is controlled by multiple genes and environmental signals (FICKLIN; FELTUS, 2013). Thus, yield improvements under stress conditions (e.g. drought) can benefit from an integrative approach, combining different levels (organ, individual plants, crop) of phenotyping, along with molecular characterization (LIU et al., 2017). Molecular events are initiated by the perception of water deficit, which is related to changes in cell volume caused by dehydration and, consequently, alterations in cell wall pressure and osmotic potential. These modifications alter the cellular structure, activating enzyme complexes that trigger cascade events, leading to the expression of several groups of genes involved in the activation of defense responses. Transcriptional factors (TFs) and elements sensitive to dehydration, such as DREB, NAC (NAM, ATAF and CUC), MYB and other families, activate downstream stress responses that are specific according to the condition. Thus, considering the complex genetic network that the stress response comprises, a critical point is to define the key genes regulating this response, in order to find a relation between tolerance to water stress and the genetic mechanisms (DIAS et al., 2016; ULLAH et al., 2018; JANGALE et al., 2019).

In this context, the identification of a cis-action dehydration responsive element (DRE), whose function is important for the expression of genes related to dehydration in Arabidopsis thaliana, was an important step in the identification of genes responsive water stress (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000). Different studies suggest that the DREB transcriptional factors have a specific function in responses to water, salt, thermal, and low temperature stress. DREB proteins can be divided into six subgroups (A1 to A6), and most *DREB1* and *DREB2* genes are involved in responses to abiotic stresses (LATA et al., 2011; KIDOKORO et al., 2015). Therefore, the aim of this study was to analyze the relative expression of Soybean DREB genes from plants submitted to different levels of water deficit.

MATERIALS AND METHODS

Plant material

Soybean plants of M9144RR cultivar were cultivated during the dry season in the experimental station of the Palmas Campus of the Federal University of Tocantins. Plants were submitted to water deficit periods during the vegetative

and reproductive phases, according to the following treatments: (1) water deficit during the entire developmental cycle, being irrigated with 25 % of the crop potential evapotranspiration (ETc) during the vegetative (V) and reproductive (R) periods (25V25R); (2) control plants cultivated in the absence of water deficit, being irrigated with 100 % of the ETc during the entire experimental period (100V100R).

Leaf samples were collected at two phenological stages, vegetative (V4) and reproductive (R4), with three biological replicates for each treatment. The samples were collected and immediately frozen in liquid nitrogen and then stored at - 80 °C. All laboratory analyses were carried out in the Laboratory of Molecular Analysis of the Federal University of Tocantins.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the leaves using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. 5 µg of total RNA from each sample were treated with Ambion® Turbo DNA-free kit (Ambion) to remove any contaminating genomic DNA. The quantity and purity of total RNA were assessed using a spectrophotometer (Nanodrop® ND-1000), while RNA integrity was visually analyzed in 1% agarose gel. The cDNA was synthesized from 1.0 µg of DNA-free RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), following the manufacturer's protocol. After the cDNA synthesis, samples were stored at - 20 °C.

Primer design

Gene expression analysis through RT-qPCR consisted in the evaluation of the expression profile of the genes *GmDREB1* and *GmDREB5*, which are genes related to water deficit (MARCOLINO-GOMES et al., 2015). The *GmDREB1* and *GmDREB5* primers (Table 1) were designed using the OligoPerfect software (https://apps.thermofisher.com/apps/oligoperfect/), and their quality was evaluated through the OligoAnalyzer tool (https://www.idtdna.com/calc/analyzer). RT-qPCR primers (Table 1) were designed from the sequences *GmDREB1*, *GmDREB5*, *GmFYV*E (MARCOLINO-GOMES et al., 2015), and *Gmβ-actin* (FUGANTI-PAGLIARINI et al., 2017) genes of *Glycine max* (L.) Merrill, using the program OligoPerfect (https:// apps.thermofisher.com/apps/oligoperfect/).

Quality assessment of the primers was performed using the and OligoAnalyzer tool (https://www.idtdna.com/calc/analyzer). The reference genes used in this study were *Gmβ-actin* and *GmFYVE*.

Table 1 - Primer sequences used for the analysis of gene expression through RT-qPCR. Fw = Primer forward, Rv = Primer reverse.

Gene	Access number	Primer sequences	Meltingtemperature (°C)	Amplicons ize (bp)
GmB-Actin	GMU60500	Fw: GAGCTATGAATTGCCTGATGG	53.6	118
		Rv: CGTTTCATGAATTCCAGTAGC	51.7	
GmFYVE	Glyma13g17500	Fw: TTCTGTCTTCTGCAAGTGGTG	55.2	92
		Rv: GATCCCTCATCCATACATTTCAG	53.0	
GmDREB1	AW308782	Fw: CCCTGAGCTCTCATCTTCCTTGG	59.0	240
		Rv: ATAGTCCCCAGCCAAATCCT	55.8	
GmDREB5	Glyma12g33020	Fw: TTGCCTACTACTACTCCTATATTCATTTCC	55.6	86
		Rv: CCTTGAAATACACGGAGCCTTAG	55.4	

Source: author's own elaboration

Gene expression analysis

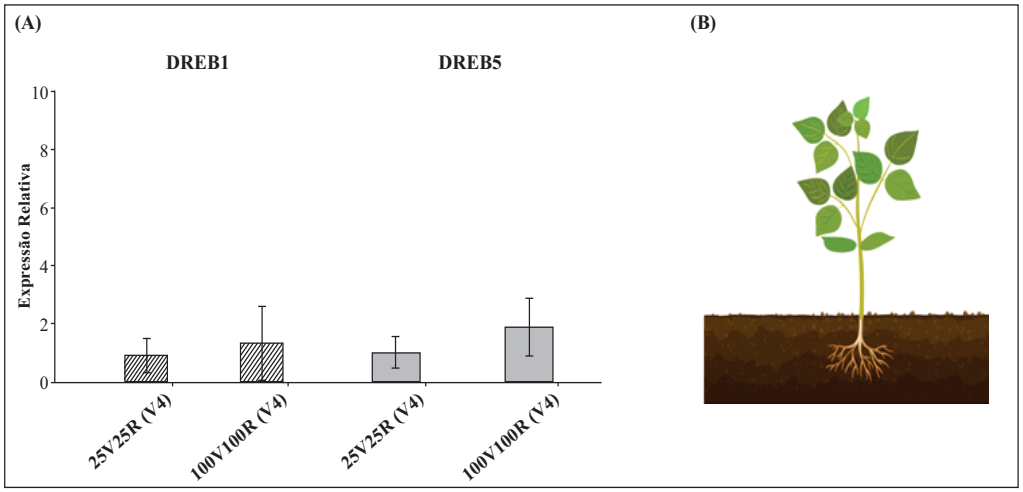
RT-qPCR analyses were carried out on a ABI PRISM 7500 fast Real-Time PCR thermalcycler (Applied Biosystems), using SYBR-green detection system and the cDNA obtained from the RNA extracted from the soybean leaves. Reactions were carried out in 10 µL reaction volume: 5 µL of SYBR-green (Master Mix PowerUp SYBR green UDG with ROX) (Invitrogen), 0.2 µL (0.2 M final concentration on the reaction) of forward and reverse gene-specific primers (see Table 1 for primer sequences and gene amplification efficiencies), 1 µL of cDNA (80 ng), and 3.6 µL of nuclease-free water. Three biological replicates for each treatment were used, reactions were run in triplicates, and amplification was performed with the following reaction conditions: 2 min at 50 °C, followed by 10 min at 95 °C, then 40 cycles 95 °C for 15 s followed by 1 min at 60 °C, and completed with a melting curve analysis to access specificity of the reaction by raising the temperature from 60 to 95 °C, with 1 °C increase in temperature every 5 s. Relative fold differences were calculated based on the ΔΔCT method (PFAFFL, 2001), using *Gmβ-actin* (FUGANTI-PAGLIARINI et al., 2017) and *GmFYVE* (MARCOLINO-GOMES et al., 2015) as reference genes, and were calculated relative to a reference sample (25V25R/V4 and 100V100R/R4).

Normalization was performed using the equation ΔCTtarget = CT (target gene) − CT (endogenous control) and ΔCTreference = CT (target gene) − CT (endogenous control). The calibration was determined by the formula EtargetΔCTtarget and EreferenceΔCTreference, where E is the efficiency value of the primers used. The calibrator was a sample used as the basis for comparative expression results. The relative quantification was obtained by the formula EtargetΔCTtarget/ EreferenceΔCTreference (PFAFFL, 2001).

RESULTS AND DISCUSSION

The *GmDREB1* and *GmDREB5* expression patterns during the vegetative stage V4 showed that, even under water deficit conditions (25V25R), these genes were not differentially expressed when compared to soybean plants under well-watered conditions (100V1100R) (Figure 1 A). This result may be associated with the phenotypic plasticity of the soybean crop and the source and sink relation observed during the V4 stage, when plants displays only three fully expanded trifoliate leaves (Figure 1 B).

Figure 1 – Relative quantitative expression profile of *GmDREB1* and *GmDREB2* in leaves from soybean plants at the vegetative stage V4 submitted to water deficit conditions, plants irrigated with only 25 % of the ETc (25V25R), and well-watered conditions, plants irrigated with 100 % of the ETc (100V100R). Columns represent the fold difference in gene expression among the different RNA extraction methods for each tissue, relative to a reference sample - 25V25R/V4. The relative quantification was obtained by the formula EtargetΔCTtarget/ EreferenceΔCTreference (PFAFFL 2001), using *Gmβ-actin* and *GmFYVE* as reference genes. Expression values for each biological sample were obtained from three biological repetitions and error bars represent the standard error for them (A). Representation of a soybean plant at the vegetative stage V4 (B).



Source: author's own elaboration

When water deficit occurs during the initial phases of development or seedling establishment, it becomes a limiting factor for soybean development, since it has a great potential to affect physiological and biochemical processes, such as photosynthesis, respiration, nutrient metabolism, growth factors, among others (JALEEL et al., 2009; BARBOSA, 2017).

According to Gava et al. (2016), the presence of moderate or severe water deficit during the phases of vegetative development, flowering, and fructification onset, impair plant growth, though it does not affect plant productivity. Modifications on the photosynthetic process can directly reflect on the agronomic features, such as plant height, stem diameter, and leaves and stem fresh and dry weight. Reductions on the photosynthetic rate can lead to decreases in the production of photoassimilates, which would be used for growth and development, potentially reducing seed production. In order to escape water deficit, plants have the ability to accelerate their life cycle and thus avoid that their organs face water deficit (BIANCHI; et al., 2016).

At the molecular level, we verified that there were no differences in the expression of the genes analyzed, however, Medina et al. (2019) observed significant differences on the expression of DREB1 and DREB2 in wheat plants when submitted to water deficit conditions, suggesting that these genes display an important role in the integration of plant responses to their growth conditions and in the co-regulation of water transport.

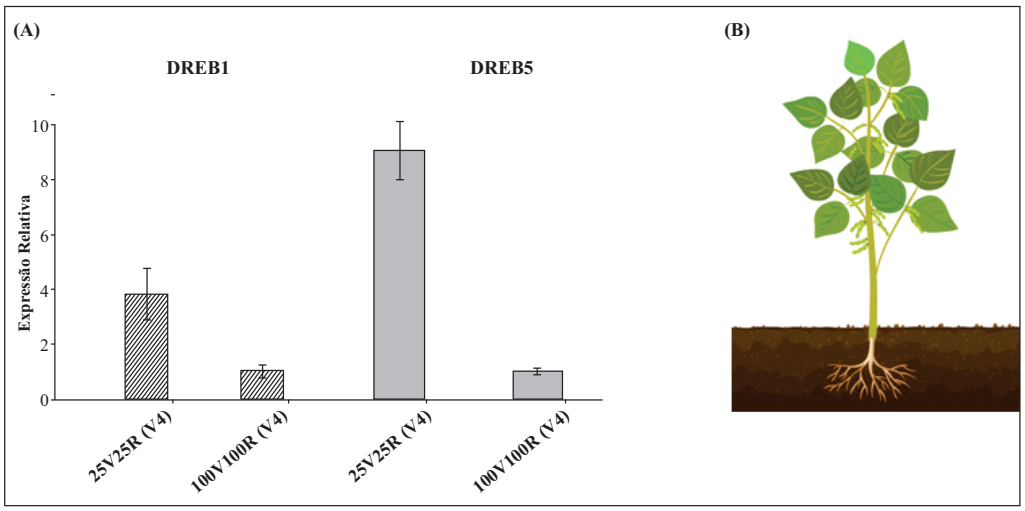
Hoekstra et al. (2001) suggest that during water deficit, different protection mechanisms are activated in different stages. As a consequence, a plant defense gene network is activated. However, the activation of these genes is associated to the stomatal and physiological differences among water deficit tolerant and susceptible varieties (JANGALE et al., 2019). A physiological characteristic that was observed by Shi et al., 2017, was that DREB1 expression inhibited plant growth, though this finding was not observed by Feng et al. 2019 through the expression of ZjDREB1.4 during the growth phase.

Water deficit periods during vegetative growth reduces growth, but during this developmental phase there is no yield components, thus, the effects on grain production are reduced and when the water conditions return to more favorable conditions, enables satisfactory levels of grain yield. (BERGAMASCHI et al., 2006)

The expression analysis of *GmDREB*1 and *GmDREB5* during the R4 reproductive phase showed that relatively higher expression levels were observed in plants submitted to water deficit conditions (25V25R). *GmDREB1* and *GmDREB5* were four and eight times more expressed under water deficit conditions, when compared to well-watered (100V100R) plants (Figure 2 A). This response is associated with the molecular responses that plants activate to deal with water deficit conditions during such an important developmental

stage (R4), when pods are completely formed and plants require a greater amount of water (Figure 2 B).

Figure 2 - Relative quantitative expression profile of *GmDREB1* and *GmDREB2* in leaves from soybean plants at the reproductive stage R4 submitted to water deficit conditions, plants irrigated with only 25 % of the ETc (25V25R), and well-watered conditions, plants irrigated with 100 % of the ETc (100V100R). Columns represent the fold difference in gene expression among the different RNA extraction methods for each tissue, relative to a reference sample (100V100R/R4). The relative quantification was obtained by the formula EtargetΔCTtarget/ EreferenceΔCTreference (PFAFFL 2001), using *Gmβ-actin* and *GmFYVE* as reference genes. Expression values for each biological sample were obtained from three biological repetitions and error bars represent the standard error for them (A). Representation of a soybean plant at the vegetative stage R4 (B).



Source: author's own elaboration

Depending on the stage of development in which water deficit occurs, soybean plants can respond in different ways. When water stress is present at the beginning of soybean flowering, the number of pods is reduced; when it occurs after the beginning of flowering and during the flowering period, the number of pods, as well as the size of the seeds, are reduced. Souza et al. (2013) observed that, when water deficiency occurs during the R5 development stage, the number of grains per pod, as well as their weight, is affected, the same observed by Nunes (2015), where the agronomic characteristics presented the lowest averages when subjected to severe water stress throughout the cycle. The results of the study by Souza et al. (2013) showed that when water deficit occurs at the beginning of flowering (R1) and during the period in which the

pods are fully developed (R4), seed production is significantly reduced when compared to the phase in which grain growth is slowed down. completed (R6) and the maturing process begins (R7).

One of the plant responses to extreme conditions is the reduced growth rates and fastening of the reproductive process, modifications that are related to the high air temperature and low water availability, which increase the respiration rates (EMBRAPA, 2011).

Medina et al. (1997) observed that soybean cultivars reduced the days for maturation when sown at late times, a feature also observed by Amorim et al. (2011), who also analyzed the effects climatic variations, such as modifications in temperature and precipitation, on the number of days needed for completing maturation. The soybean crop requires around 450 to 800 mm of water during their life cycle for obtaining the maximum yield levels (EMBRAPA, 2011). The water requirement increases during soybean development, reaching maximum levels during flowering and grain filling, when plants need seven to eight mm per day, decreasing after these phases of the life cycle. Water stress during these phases can cause significant physiological problems that can lead to premature leaf shedding and, consequently, reduction in productivity levels (GAVA; RICARDO, 2014).

The difference in the expression pattern of *GmDREB1* and *GmDREB5* in response to water deficit illustrates how one or more mechanisms can be activated to deal with water deficit conditions during the period that soybean plants most require water. A physiological relation that can be observed in the M 9144 RR cultivar under stress conditions is the fact that stomatal closure. Stomatal closure during water stress conditions occurs in response to the hormone Abscisic Acid (ABA), which is seen as a cornerstone of the stomatal function, since it acts as a trigger that activates guard-cell membrane channels and transporters, leading to the reduction of guard-cell turgor and, consequently, to stomatal closure (BAUER et al., 2013).

Fuganti-Pagliarini et al. (2017), state that genes related to water deficit have higher levels of expression in drought conditions, showing that plants can change their metabolism in response to adverse environmental conditions, allowing a higher rate of survival and/or maintenance of productivity levels. Stolf-Moreira et al. (2010) found, in an experiment with different soybean cultivars, an increase (two to three times) in the expression levels of *GmDREB1* under conditions of water deficit. In addition, they point out that some genes induced by water stress encode proteins that can confer tolerance to adverse conditions.

Zhou et al. (2018) observed that many soybean genes, from different pathways, are differentially regulated under water stress conditions, indicating that soybean plants activate complex mechanisms to deal with water stress.

Marcolino-Gomes et al. (2013), have identified highest expression levels for GmDREB5 in root and leaf tissues, in both soybean cultivars analyzed in drought stress. Le et al. (2012), observed that two soybean genes, orthologues of the Arabidopsis genes *DREB1A* and *DREB1D*, from the AP2_EREBP family, and the gene ZAT 10 / STZ, from the C2H2_Zn family, were induced in leaves, from plants submitted to water stress conditions, during the vegetative stage, but not during the reproductive stage, indicating that these TFs may be involved in the response regulation to water deficit during vegetative growth. These studies show that water deficit triggers responses both conserved and specific depending of the developmental stage and/or intensity of the water deficit.

CONCLUSION

The expression analysis of *GmDREB1* and *GmDREB5* show that water deficit induced their expression during the reproductive stage, showing that different mechanisms may be activated to deal with water deficit conditions during the period that soybean plants most need water supply.

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