Article

Evaluation of Some Defense Gene Expression in Soybean Cultivars under Drought Stress

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Abstract: Drought stress on soybean is a research-demanding matter for negative influence that agricultural drought brings about. This study was designated to evaluate the effect of drought stress on some gene expression in flowering and pod elongation stages in soybean. This experiment was carried out in split-plot format with RCBD design with four replicates. Drought stress as the main factor included three levels (irrigation after 50, 100, and 150 millimeters evaporation from the A-class evaporation pot) of which 50 millimeters evaporation is considered as control. The sub-factor included a factorial combination of 3 varieties (DPX, Sari and WE6) and two sampling stages (flowering and pod elongation). The gene expression analysis was carried out by using the QRT-PCR technique. According to our results, all genes have shown overexpression in drought stress despite this result was not the same for all genotypes and stress levels. Some genes have up-regulated in mediate stress (treatment 100) level (like as *Gmdreb 2*, *Gmdreb 5*, *GmRD20A*, *GmaxACD2*) and other genes up-regulated in serve stress (treatment 150) level. Between genotypes, DPX cultivar and WE6 line were better than of the sari cultivar for all genes up-regulated.

Keywords: Flowering; Gene expression; Pod elongation; Soybean; Water deficit

Introduction

Drought stress serves as one of the most important limiting factors on crop production that may be occurred in any stage of crop growing season. The crops may encounter water deficiency frequently in their lifespans. However, it's found to be most damaging in some specific phenology stages such as germination, seedling, and flowering [1,2]. Soybean as a legume growing in the Mediterranean climate is known to be a very economically important commodity due to its high nutritional value (40% protein and 20% oil). It belongs to the crop group which has low drought tolerance. Thus, selective breeding high drought-tolerance soybean cultivars and studying methods to improve the drought tolerance of soybean are interesting topics for many breeders. [3]. Plants generally respond to their changing environment in a complex, integrated path allowing them to respond and adapt to the specific complex of conditions and constraints present at a particular time. [4]. Drought stress induces the expression of various genes that are involved in stress tolerance and response [5]. Evidence exists to illustrate the presence of both ABA-independent and ABA-de-

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pendent genes regulatory systems controlling drought-inducible gene expression. Both cis-acting and trans-acting regulatory elements are operative in ABA-independent and/or ABA-dependent gene(s) expression induced by drought stress has been exactly analyzed at the molecular level [6]. These genes have important roles in the resistance of plants to drought stress by identifying the dehydration-responsive element. Some of the DREB members (dehydration responsive element binding protein) gene subfamily are present in the soybean genome (such as *GmDREB1*, *GmDREB2*, *GmDREB3*, *GmDREB5*, and …) [7]. Chen et al [8] introduce a novel DREB homologous gene, the *GmDREB2*. This gene was originally isolated from soybean crop and belong to the A-5 subclass in the DREB group in the AP2/EREBP family. According to Chen, this gene group showed over-expression under drought, salt, and low-temperature stress [8]. In addition, there are some genes classified into the *GmDREB5* that were identified through an insilico approach from soybean. This gene group was homologous to the Arabidopsis *ERD1*, *RD20A*, and *RD22* genes [9]. These genes are typical markers for the ABA-dependent and ABA-independent pathways of response to drought [10]. Guimarães-Dias et al [11] reviewed some of the genes (such as *GmaxLKR/SHD-like1*, *GmaxLKR/SHD-like2*, *GmaxADC2*, *GmaxGLOS2-like1*, *GmaxGLOS2-like2*, and *GmaxGLOS2-like3*) in response to drought stress. Evidence suggests that the soybean is much more susceptible to drought stress in the early stages of reproduction (flowering period to early pod development) compared to other stages of plant development [12]. The response to drought stress relies mainly on gene expression regulation, of thousands of genes, and then in this study, we aimed to evaluate the effect of drought stress on some genes expression involving drought-tolerant mechanisms in different soybeans genotypes.

Materials and Method

Plant material and drought assay

This experiment was conducted in an agriculture research center in Golestan province arranged in a split-plot and randomized complete block design (RCBD) with four replicates. As a main factor drought stress included three levels (irrigation after 50, 100, and 150 mm evaporation from the evaporation pan A class) of which 50 millimeters evaporation was considered as control. The sub-factors included the factorial combination of three cultivars (DPX, Sari, and WE6) and two growth stages (flowering and pod elongation). The plant was grown in the field and irrigation was carried out based on evaporation from the evaporation pan A class for each plot. In different stages of the plant, the development included flowering and pod elongation, to measuring gene expression 5 gr leaves removed in different treatments were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Total RNA isolation, DNase Treatments, and Reverse transcription

RNA extraction was done by Biozol Reagent (Bio PLUS, Japan). RNA samples were treated with RNAse-free DNase I (Biolabs) to eliminate any DNA contamination. Total RNA was checked by electrophoresis on a 1.5% agarose gel (Fig 1), and RNA concentration was determined using a NanoDrop spectrophotometer BT-600 (Thermo Scientific). the first-strand cDNA was then generated from 1 μg of template RNA and tested by housekeeping gene primers using PCR. The specific function of primers used by cDNAs in a standard polymerase chain reaction was evaluated.

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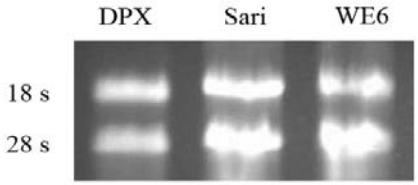

Fig 1-RNA band isolated from soybean Genotype.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Primers were designed based on the information available on the NCBI site using primer software 3 [13]. RT-qPCR reactions were carried out by the IQ5 machine used by Bio-Rad Company and Cyber Bio Pars Kit (Gorgan University of Agricultural Sciences and Natural Resources) that was able to evaluate in real-time. As housekeeping genes, the Actin gene was used for the normalization of target gene expression. The sequences of the 11 primers used in the RT-qPCR analysis are listed in Table1. At the end of the reaction, after receiving the charts, the information was transferred to the REST software and the data were analyzed. The illustrations were drawn using Excel 2016 software.

Table 1- primer information

Genes	Forward primer sequence [5′3′]	Reverse primer sequence [5′3′]	Amplicon length (pb)
GmActin	CTGATCGCATGAGCAAGGAA	GGGGATGTTGAGAATAGCAGGA	175
GmDREB2	ATGGAAGAAGCGTTAGGTGGAGA	TGGAGGACGTCGAGTATTGTGG	415
GmDREB5	AACAGCAGCATCAGCAGCAC	CGGATTTCAGCGACCCATT	199
GmaxRD20A	GTGGCACATGACTGAAGGAA	ATCTTTCCAGCAGCACCTCT	195
GmaxRD22	AATGCCGAAAGCCATTACAG	GCTTTGTTTTCCCTGCGTTA	110
GmaxERD1	CGTCCAGAATTGCTCAACAG	TGGGGTTATAGCCTTGTTGG	184
GmaxLKR/SDH1	ATCCTGCCACCTACAAATGG	ACGGAAAATGGTTGATGCTT	182
GmaxLKR/SDH2	GGGGAATGGTGTGATATGCT	ATTGGCTATGCAAGCTCTCC	166
GmaxADC2	CAGGAGTATGTCAGCCACGA	CAGATCTTGAGCAGCAGGAA	144
GmaxGOLS2 like-1	CCTGAGAACGTTGAGCTTGA	CCACCACTTCTTCACCAACA	132

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GmaxGOLS2 Like-2	AGTCACCACTCCCACTTCGT	CCCGTATATCTCCACGGTTT	192
GmaxGOLS2 Like-3	TTGCCATGGCTTATTACGTC	TACCTCAATGTCTCCGTCCA	98

Result

The expression of the *GmDREB2* gene is shown in figure 2. The results showed that the expression of this gene in different cultivars was affected by drought stress as an increase at the first drought level (treatment 100) and decreased with increasing drought stress at the second drought level (treatment 150). In both stages of flowering and pod elongation, the highest amount of expression of this gene in the first level of drought stress (treatment 100) was observed. Among the cultivars, Sari showed the lowest expression of *the GmDREB2* in all stages, which could indicate the higher sensitivity of this variety to drought stress than the other two genotypes.

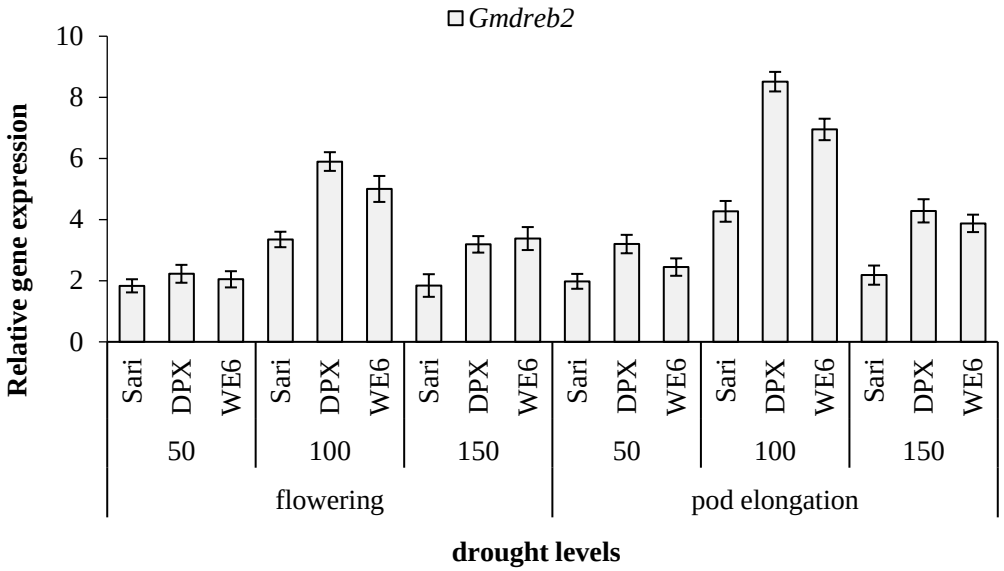

Fig 2- *GmDREB2* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

The GmDREB5 gene expression results (Figure 3) showed that the expression level of this gene under drought stress did not significantly increase at the flowering stage but in the second level of stress (treatment 150), its amount decreased compared to the control's (treatment 50), although the difference between them was not significant. In the pod elongation stage, the expression level of *the GmDREB5* gene increased at the first level of drought stress (treatment 100) but decreased with the increase of drought stress intensity at the second level (treatment 150). The highest expression of *DREB5* expression was observed in the DPX cultivar at the pod elongation stage and in the first level of drought stress (treatment 100), which was not significantly different from the expression of this gene in the WE6 line.

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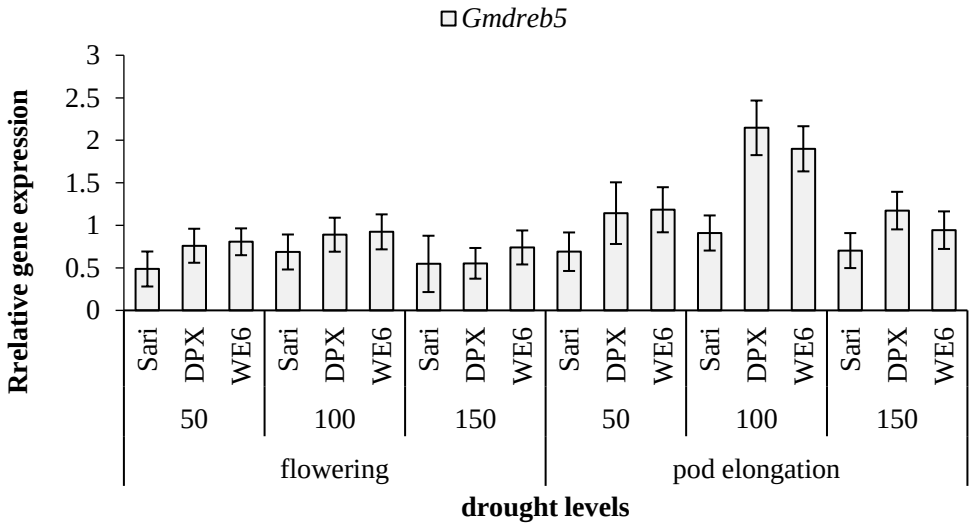

Fig 3- *GmDREB5* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

As seen in figure 4, the expression level of *the GmRD20A* gene increased with the induction of drought stress in both stages of flowering and sheathing, which increased in the first level of stress (treatment 100) and had a significant difference with other stages. The WE6 line in the pod elongation stage and the first level of stress (treatment 100) showed the highest expression of this gene and its difference with other treatments was significant.

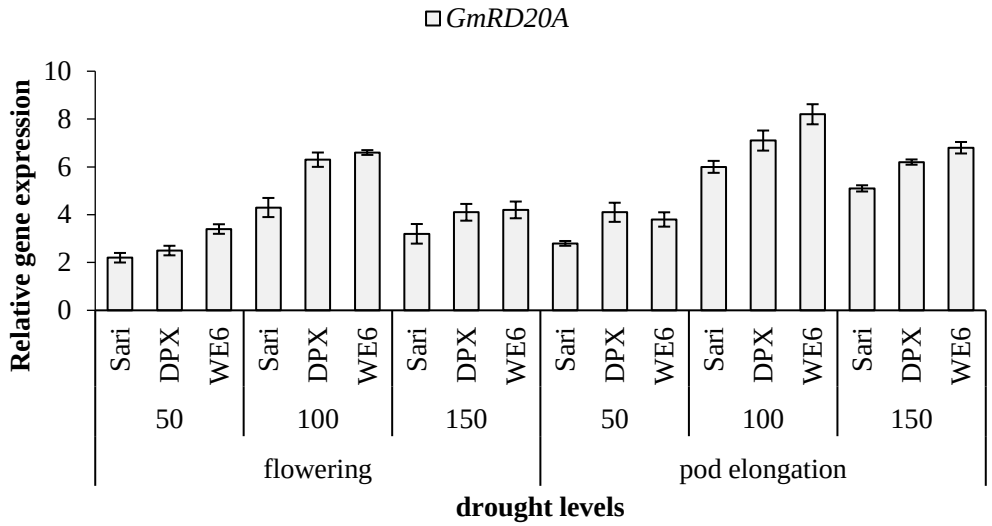

Fig 4- *GmRD20A* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

The expression of the *GmRD22A* gene is shown in Fig.5. The expression of this gene increased in the second level of drought stress (treatment 150) at both stages (flowering and pod elongation), and there was a significant difference with other treatments. at both stages, WE6 line higher expression of the *GmRD22A* gene showed that its difference was significant under drought stress levels with two other genotypes in the pod elongation stage but at the flowering stage did not significantly differ with DPX.

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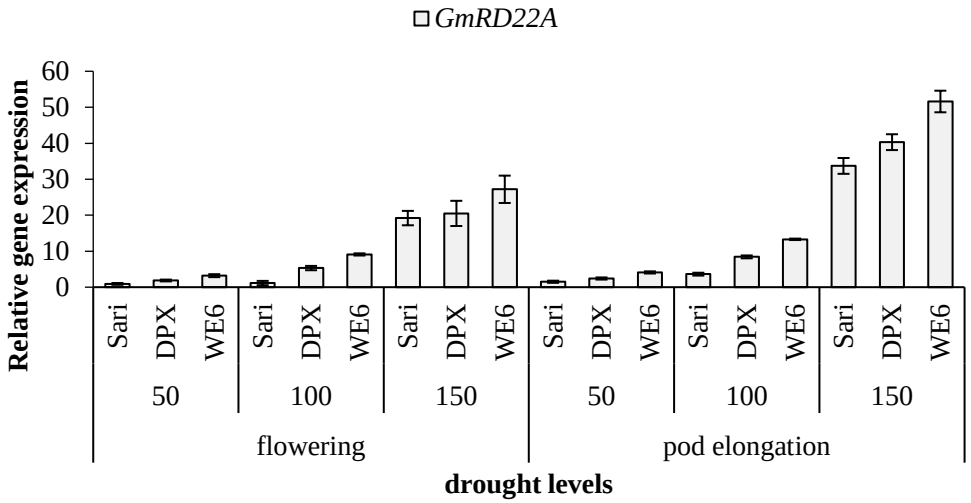

Fig 5- *GmRD22A* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

As shown in Fig.6, the expression of *the GmERD1* gene was influenced by drought stress and increased expression. At the flowering stage, the DPX cultivar had the highest expression of *GmERD1* gene expression in control (treatment 50) and the first drought stress level (treatment 100), which was increased at the second level of stress (treatment 150) but its amount was expressed as the amount of expression of the gene in the line WE6, was less. In the pod elongation stage, the expression of the *GmERD1* gene in all genotypes was significantly increased at the first level (100 treatments), but in the second level of drought stress (treatment 150), its expression for the Sari cultivar decreased and increased for the WE6 line and the DPX variety.

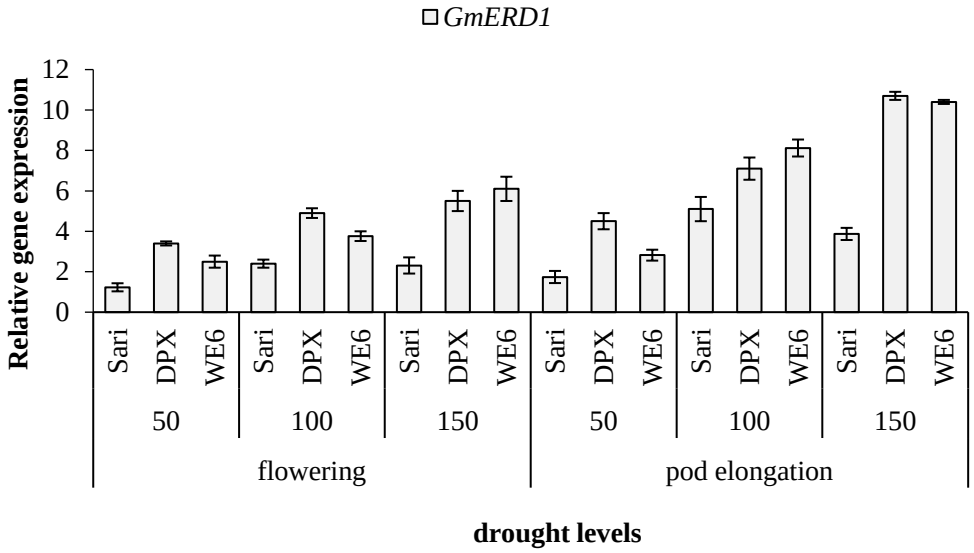

Fig 6- GmERD1 gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

The *GmaxLKR/SHD-like1* gene expression (Fig.7) in all three cultivars compared to control (treatment 50) showed a higher expression, so that in the second level of drought stress (treatment 150), at both stages (flowering and pod elongation), the highest amount of this gene was observed in all genotypes. The DPX cultivar had shown higher expression

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of drought stress and its difference with the other two genotypes was significant. Similar conditions were observed in the expression of the *GmaxLKR/SHD-like2* gene expression (Fig.8). The results showed that the expression of both genes was higher in the pod elongation stage compared to the flowering stage.

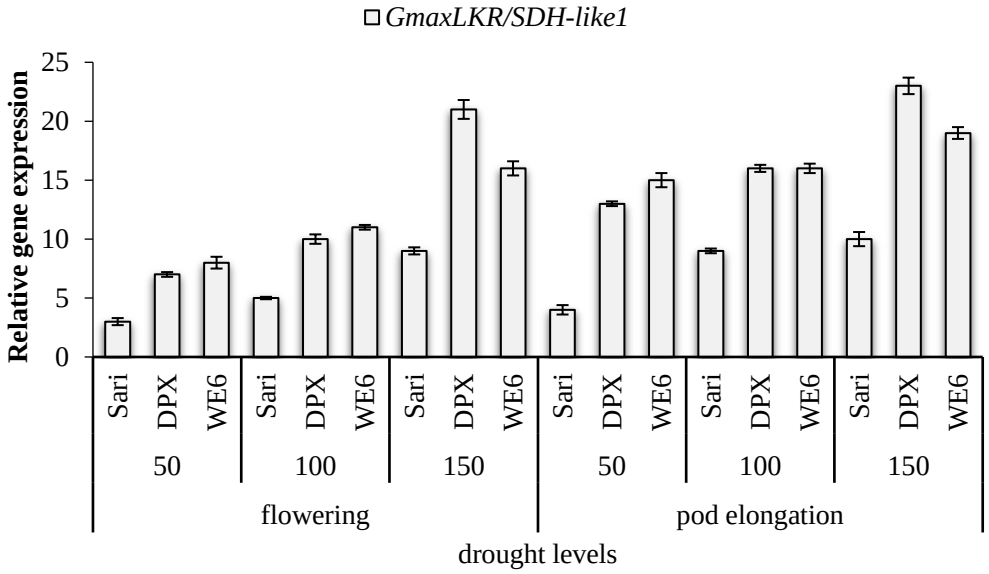

Fig 7- *GmaxLKR/SHD-like1* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

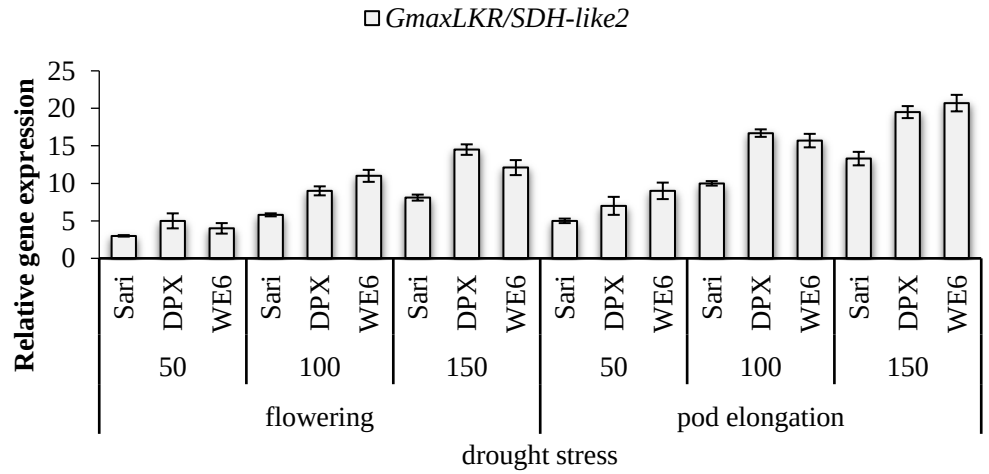

Fig 8- *GmaxLKR/SHD-like2* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

GmaxADC2 gene expression results (Fig.9) showed that the expression of this gene up-regulated in all three genotypes at the flowering stage so that the WE6 line showed the highest expression at the first level of drought stress (treatment 100) and it was significantly different from the other genotypes. But in the second level of drought stress (treatment 150), the expression of *GmaxADC2* in the WE6 line did not significantly increase and the difference was not significant in comparison with the DPX cultivar. In the pod elongation stage, the expression of *GmaxADC2* increased at the first level of drought stress treatment (treatment 100), and in the second level (150 treatments) its amount decreased,

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but its amount did not decrease rather than the expression of this gene in the control treatment (treatment 50). At all levels of drought stress, WE6 had the highest amount of expression, except in the control treatment (treatment 50), which had a significant difference from other genotypes; under drought stress conditions the difference was not significant in comparison with DPX.

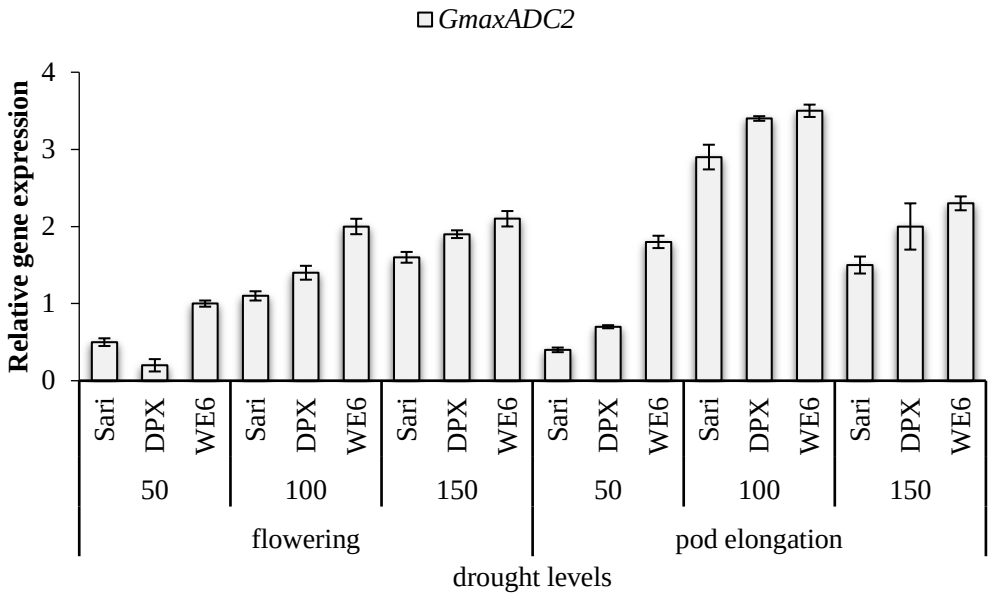

Fig 9- *GmaxADC2* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

As shown in Fig.10, the amount of *GmaxGLOS2-like1* gene expression increased under drought stress, and in the second level of drought stress (treatment 150), in both stages of flowering and pod elongation, they showed the highest value, which did not differ significantly. A significant decrease in the amount of expression of this gene in the Sari cultivar was observed at the first level of stress treatment (treatment 100), which was observed at both stages of flowering and pod elongation, but its level in the second level of drought stress increased significantly.

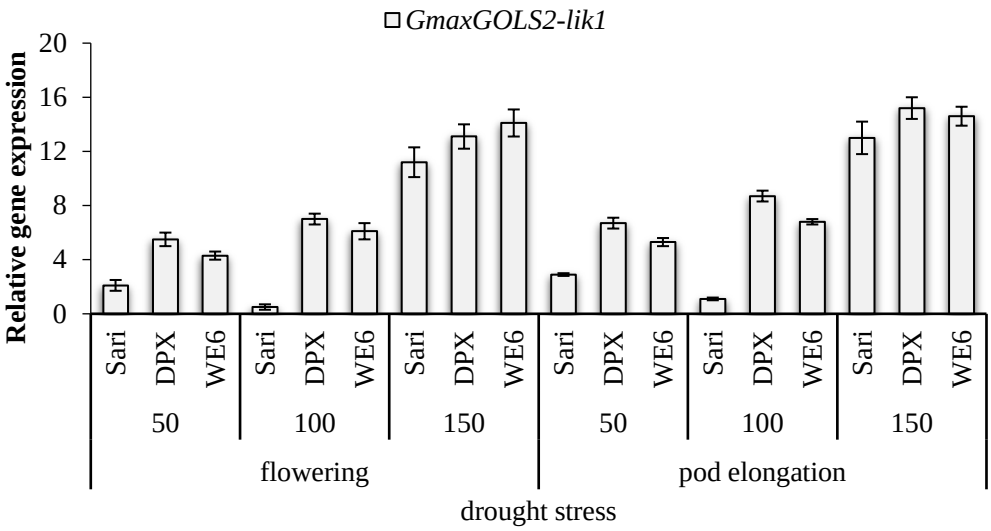

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Fig 10- *GmaxGLOS2-like1* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

The expression of *GmaxGLOS2-like2* and *GmaxGLOS2-like3* genes (Fig. 11 and 12, respectively) showed a similar trend so that in both stages of pod elongation and flowering, the first level of stress increased slightly and in the second level of stress, a significant increase was observed which was seen in the DPX and WE6 line, but the Sari did not have a high expression of these genes.

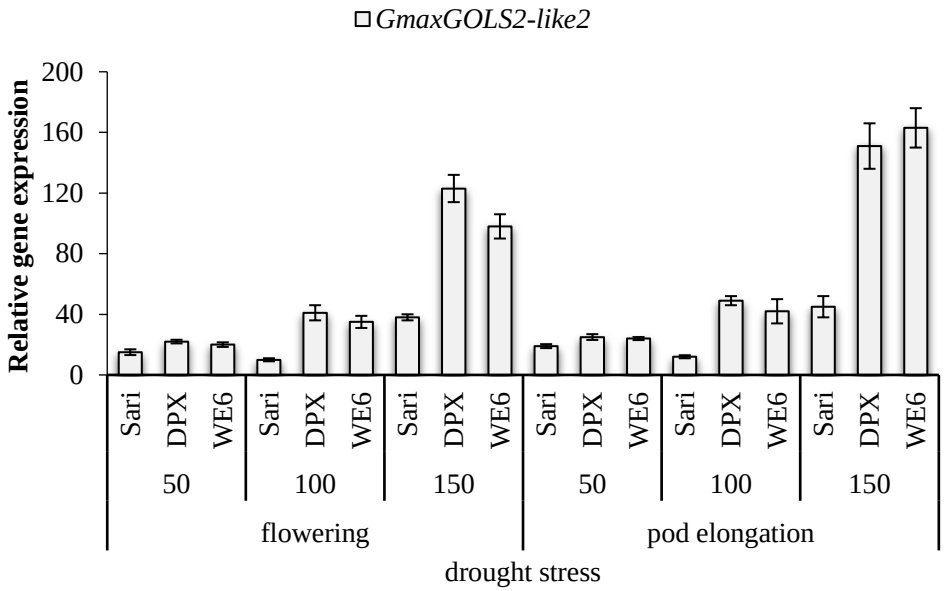

Fig 11- *GmaxGLOS2-like2* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

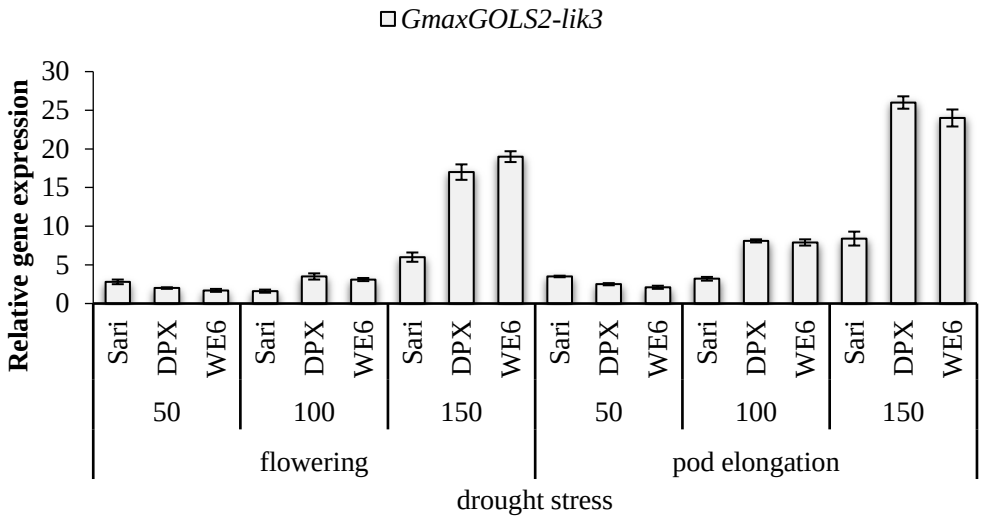

Fig 12- *GmaxGLOS2-like3* gene expression in Flowering and Pod elongation at different drought levels (50 (control), 100 and 150 mm evaporation from the evaporation pan A class).

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Discussion

About the *GmDREB2* gene at the flowering stage, our result has shown low expression rather than pod elongation, it can be indicated the greater sensitivity of this stage of plant growth to drought stress, which has been reported to be susceptible to drought stress in other researchers [14,8]. Across the DRE-binding proteins, the *DREB2* subfamily is induced by drought stress indicating their main role in stress-responsive gene expression. [14]. In the same study, Chen et al [8] reported expression of *the GmDREB2* gene induced the expression of *Rd29A* and *cor15a* genes, that already have been identified as downstream genes of *AtDREB1A* in Arabidopsis, and hence enhanced their tolerance to drought and high-salt stresses. Overexpression of *the GmDREB2* gene activated the expression of some downstream genes involving free proline biosynthesis that function as an osmolyte in the stress tolerance of plants, which in turn, increased tolerance to drought stresses in transgenic plants [15]. To date, there have been a few reports about the isolation of *the GmDREB5* gene from soybean. In the first study about the *GmDREB5* gene, Chen et al [8] isolated this gene from soybean cultivars with 927 bp in length, and in another study, presented some results on amplification and characterization of *GmDREB5* gene from mRNA isolated from soybean cultivar Xanh Tendai in Vietnam [3]. Previously some researchers [16,17] reported the *Gmdreb5* gene overexpression under drought stress and oscillation during the day, like other DREB subfamily.

Generally, the expression of the *GmRD20A* gene in all three genotypes and all three treatments in the pod elongation stage was more than in the flowering stage, which could indicate a higher sensitivity to drought stress. In the same study, Neves-Borges et al [9] reported the *GmaxRD20A-like* showed a similar expression profile in the sensitive and tolerant soybean cultivars. Although, *GmaxRD20A-like* was more expressed in the tolerant cultivar under intense stress. In Arabidopsis, the *RD20A* gene is regulated by the AREB1/ABF2 transcription factor, which is too involved in the regulation of the *RD29B* gene [18,19].

GmRD22 is an apoplastic protein, it may play a regulative function in cell wall metabolism through interacting with other cell wall proteins; which protein affects cell wall integrity and reduces salinity and osmotic stress in plants. [12,20]. Wang et al [12] declare that the *GmRD22* can induce the expression of cell wall peroxidases and improve cell wall integrity under stress conditions. Neves-Borges et al [9] reported that *GmaxRD22*-like gene expression was extremely induced in plants grown under intensive stress.

The gene ERD1 (Early Responsive to Dehydration) with encodes a chloroplastic ATP-dependent protease has an important role in soybean responsibility to drought stress [21]. This gene is induced quickly by drought stress, the encoded protein has a variety of structural and functional roles in order to switch the stress response signaling pathway. More specific characterization of the *ERD* gene group showed that they present different and heterogeneous biochemical functions and are exhibited in vast subcellular components [22]. In similar research, the GmaxLKR/SDH-like1 and GmaxLKR/SDH-like2 soybean genes represented the same expression regulation. It seems that the respective promoter regions may not have diverged among the duplicated genes. Nevertheless, these genes showed a rather distinct gene expression profile between sensitive and tolerant varieties in the cultivation condition [11]. The saccharopine pathway is the basic irreversible pathway for lysine catabolism in higher eukaryotes [23]. Lysine-ketoglutarate reductase (LKR), which condenses lysine and a-ketoglutarate into saccharopine and saccharopine dehydrogenase (SDH), which hydrolyzes saccharopine into a-aminoadipic-d-semialdehyde (AASA) and glutamic acid, and encoding by *LKR/SHD* genes. These genes are encoding LKR/SDH and AASADH persuaded in response to osmotic and oxidative stresses in the plant [24]. But the mechanism by which the enzymes protect against this stress remains to be illuminated [23].

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ADC Gene produces the Arginine decarboxylase (ADC) enzyme and plays a role as a key plant enzyme that converts arginine into putrescine, an important moderator of abiotic stress tolerance [25]. Whereas the first reportage described putrescine accumulation under potassium deficiency decades ago changes in Polyamine levels have been universally observed in different plant species exposed to a wide range of abiotic stresses, including drought, salinity, heat, cold, and others [26].

The main enzyme in the synthesis of raffinose subfamily oligosaccharides is Galactinol synthase (GolS) which acts as an osmoprotectant in plant cells [27]. Galactinol, a galactosyl derivative of Moy-inositol, acts as a galactosyl donor in the biosynthesis of Raffinose Family Oligosaccharides (RFOs). The most popular RFOs actual in plants are raffinose, stachyose, verbascose, and ajugose. Although RFOs are generally characterized as compatible solutes as a section of stress tolerance mechanisms [28], they also participate in multiple desperate plant cellular functions including transport and storage of carbon, signal transduction, membrane trafficking, mRNA export, osmoprotectants during seed desiccation, cellular Reactive Oxygen Species (ROS) homeostasis and phloem-mobile signaling syntax under stress [29].

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