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STUDY OF THE EXPRESSION OF CATALASE (CAT), ASCORBATE PEROXIDASE (ASCP), GLUTATHIONE REDUCTASE (GR) GENES IN SOYBEAN DROUGHT-TOLERANT AND SENSITIVE CULTIVARS USING REAL-TIME PCR

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Mohammad Hassanvand, Shahab Khaghani, Mahdi Changizi, Masoud Gomarian, Ezatollah Sedaghatfar: Study of the expression of Catalase (*CAT*), Ascorbate peroxidase (*Ascp*), Glutathione Reductase (*GR*) genes in soybean drought-tolerant and sensitive cultivars using real-time PCR -- Palarch's Journal Of Archaeology Of Egypt/Egyptology 18(7), ISSN 1567-214x

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ABSTRACT

Drought is one of the major constraints that limit agricultural productivity. Some factors, including climate changes and acreage expansion, indicate the need for developing drought-tolerant Genotypes. for the Study of the expression of Catalase (CAT), Ascorbate peroxidase (Ascp), Glutathione Reductase (GR) genes in soybean drought-tolerant and sensitive cultivars using real-time PCR. Seeds from Karbin (drought-sensitive) and Fora (drought-tolerant) lines were planted under specific temperature conditions at two levels of normal irrigation conditions and drought stress treatment, in the research greenhouse of Islamic Azad University of Arak, Iran. Changes in gene expression compared to control samples were recorded using the formula $2^{-\Delta\Delta CT}$. Three technical replications were given for each cDNA sample related to each sampling and used to analyze test data from Excel software. The results showed that the expression threshold of (Glutathione) gene expression was lower than the reference gene in both treatments (stress treatment and normal conditions) than the reference gene expression Still resistant line had more name in stress treatment and normal conditions treatment. The Glutathione gene, like the CAT gene in the resistant this result showed that the expression of GR gene, like the expression of CAT gene in resistant line before stress treatment, was higher than the sensitive line. The theory is also correct that chaperone proteins produced during the plant growth cycle do not disappear to express the Glutathione Reductase gene. The ASCP gene expression cycle indicates that the proteins produced by this gene have a high rate of expression before stress in cells.

INTRODUCTION

Drought is one of the major constraints that limit agricultural productivity. Some factors, including climate changes and acreage expansion, indicates towards the need for developing rought

tolerant Genotypes (Liu et al. 2004) (Coutinho et al. 2019) .Oilseeds are the second-largest food reserves after cereals (Food Outlook – Biannual Report on Global Food Markets 2020). Soybean is one of the most important oil crops that in 2018 according to the Food and Agriculture Organization of the United Nations FAO in the world area of 132895834 hectares and in Iran more than 85000 hectares (http://www.fao.org/faostat/en/#data). Soybean: structure, benefits, and cultivation Soybean (Glycine max L. Merr.) Belongs to the family Fabaceae, subfamily Faboide-ae, and the genus Glycine. The genus Glycine is divided into two subgenera: Glycine and Soja based on the nature of their growth, that is, perennial or annual, respectively. The cultivated soybean belongs to the subgenus Soja and is predominantly cultivated during summer. The height of the plants varies from 0.2 m to 2 m. Soybean has trifoliate leaves, with each node of the raceme interleaved individually with flowers, a five-toothed calyx, a glabrous corolla with long-clawed petals, a keel, and seed (Dashti et al. 2016). Glycine is highly sensitive to abiotic stresses (Cao et al. 2018) (Sun et al. 2019) (Jin et al , 20_19). Drought, the occurrence of a substantial water de_cit in the soil or in the atmosphere, is an alarming constraint to crop productivity and yield stability worldwide. It is the leading environmental stress in world agriculture, causing losses in crop yield, probably exceeding losses from all other causes combined. Drought stress adversely affects various vital physiological and biochemical processes in plants, leading to reduced growth and final crop yield. Some plant species have evolved mechanisms to cope with the stress, including drought avoidance, dehydration avoidance, or dehydration tolerance. Such adaptive mechanisms result from a multitude of morphoanatomical, physiological, biochemical, and molecular changes. Osmo regulation is the most common physiological adaptation, which reduces cellular water potential via the accumulation of a variety of organic and inorganic solutes in the cell. Consequently, such plants are capable of taking up water from a low water potential medium to sustain normal or near-normal physiological processes necessary for growth and development. However, most economically important crop species lack the capability of coping with this type of drought stress, precluding their cultivation under water-limited conditions. Various strategies have been proposed to facilitate crop production under drought conditions, in particular, the development of new crop varieties with enhanced drought tolerance. Genetic improvement of crop plants for drought tolerance is a long-term endeavor, which requires, among other things, the availability of genetic sources of tolerance, knowledge of the physiological mechanisms and genetic controls of tolerance traits at different developmental stages,, and employment of suitable germplasm screening and breeding protocols. An alternative and quicker strategy to promote plant drought tolerance is an exogenous application of various compounds, including organic solutes (organic Osmolytes and plant growth regulators) and mineral nutrients. Recently, this strategy has gained considerable attention because of its efficiency, feasibility, and cost- and labor-effectiveness (Ashraf et al. 2011).

In contrast to C3 photosynthesis, the response of C4 photosynthesis to water stress has been less-well studied despite the significant contribution of C4 plants to the global carbon budget and food security. Evidence indicates that C4 photosynthesis is highly sensitive to water stress. With declining leaf water status, CO2 assimilation rate and Stomatal conductance decrease rapidly, and photosynthesis goes through three successive phases. The initial, mainly the Stomatal, may or may not be detected as a decline in assimilation rates depending on environmental conditions. This is because the CO2-concentrating mechanism is capable of saturating C4 photosynthesis under relatively low intercellular CO2 concentrations. Also, photo respired CO2 is likely to be Refixed before escaping the bundle sheath. The main non-Stomatal factors include the reduced activity of photosynthetic enzymes (Ghannoum 2009). Abiotic stresses increase or decrease the expression level of relevant genes by altering the pathway of carbohydrate metabolism, thereby inducing or inhibiting the expression of certain genes (Ceusters et al. 2016) (Amin et al. 2019). These genes are divided into two main groups: functional genes and transcription factors. Drought stress leads to an increase in the relative concentration of oxygen free radicals, especially hydrogen peroxide (Golden, Hinerfeld, and Melov 2002). Reactive oxygen species are generated by various sources from the environment and normal cellular functions (e.g., mitochondrial metabolism). Free radicals (e.g., superoxide and hydroxyl radicals), Nonradical oxygen species (e.g., hydrogen peroxide), and reactive lipids and carbohydrates. Oxidative damage to DNA can occur by many routes, including the oxidative modification of the nucleotide bases, sugars (Gracy et al. 1999) (Spoel and Van Ooijen 2014) (Foyer and Noctor 2005). Glutathione reductase is one of the most important free radical scavenging enzymes. This enzyme plays an important role in counteracting oxidative stress and balancing oxygen-free radicals. Such a role may be performed directly by an unmediated reaction or by an enzymatic mechanism by reducing the concentration of hydrogen peroxide (Noctor and Foyer 2016). There is clear evidence for the effect of H2O2 concentration on glutathione Reductase activity. This was investigated by Smith et al. (1984) in an experiment on mutants lacking the relative activity of the enzyme Catalase (the active enzyme that reduces H_2O_2) (Smith et al. 1984). In this study, increasing the concentration of H2O2 in the studied mutants was associated with increasing the concentration of Glutathione Reductase. In a supplementary experiment, a spray of compounds that inhibited the activity of Catalase on the leaves of a soybean plant by increasing the amount of H2O2 led to an increase in the concentration and activity of the glutathione Reductase. Another activity of the glutathione Reductase gene is a very important role in cell membrane protection, especially under stress (Maruyama et al. 2004) (Herbette et al. 2002). In drought stress, oxidative stress occurs first, so among these genes, the genes involved in the pathway of free radical scavenging are of great importance. Therefore, identifying the relevant candidate genes intolerant and sensitive lines by evaluating the changes in gene expression under stress conditions compared to the non-stress state can be an excellent starting point for implementing soybean resistance to stress programs. The response of these genes in the studied genotypes to drought stress is studied with the aim of selection for tolerant

genotypes. This Study expresses the genes of Catalase (CAT), Ascorbate peroxidase (Ascp), Glutathione Reductase (GR), which are defense barriers against reactive oxygen species. In this study, using real-time PCR technique, the expression of genes under drought stress and non-drought conditions in two selected genotypes of soybean was compared.

MATERIALS AND METHODS

Seeds were prepared from two lines: Karbin (drought-sensitive) and Fora (drought tolerant) from Karaj Seed and Seedling Breeding Research Institute. Seeds were sown in temperature (2±30 ° C) and 16 hours of light (2±20 ° C) and 8 hours of darkness in two levels of regular irrigation and drought stress, in the research greenhouse of Islamic Azad University, Arak city center province. The duration of drought stress was from the three-leaf stage to 7 days (Prolonged stress). Leaf sampling was performed in stage V1 (simple leaves are open enough) by observing the leaves' tubularity on the seventh day to investigate the expression of genes induced by drought stress. After sampling, the samples were placed in liquid nitrogen and stored at -80 ° C to extract total RNA. Total RNA was extracted using a Sina clone gene RNA extraction kit. Sinapure-RNA(cell culture. Tissues) PR891620-EX6031. Using a Nanodrop to determine the concentration (NanoDrop 1000 spectrophotometer), the absorbance of each sample was recorded at 230, 260, and 280 nm. CDNA (Sinnaclon First Strand DNA Synthesis Kit-50T) from Sina Clone was used to remove genomic DNA and synthesize the first strand of CDNA. 2 µl of extraction buffer was poured into each sample in a 0.2 µl Microtube. Mixed for 16 material samples and an additional unit (unit for testing) was added to the extraction solution (34 µl in total). 0.5 microliter RT (RevertAid MMuLVReverse Transcriptase) was added per sample, and an additional unit whole 8.5 µl was slowly inverted. 0.5 µl per sample was added, and one unit of RNase inhibitor was added to the microtube (total 8.5 µl) and inverted slowly for 30 seconds. 2 µl per sample and one unit more than the total dNTP mixture was added to the tube µl 34 and slowly inverted. Finally, 85 µl of DEPC Water was added for 16 samples and one more unit to the tube. The tube containing the CDNA solution was placed in Banmarry for 60 minutes at + 42 ° C. Stop the reaction at + 85 ° C for 5 minutes. Samples were stored at -20 ° C.

DESIGN OF PRIMERS

To design the primers, the gene sequences of Catalase, Ascorbate Peroxidase, and Glutathione Reductase were downloaded from the NCBI Gene Bank (National Center for Biotechnology Information, 2001). Dedicated primers were designed using Oligo7 software and the Primer 3 Plus

site (Table 1). The primers were synthesized by SinaClone Company. PCR and electrophoresis were performed to ensure the speci_c performance of the primers. PCR products were observed on 1% agarose gel to provide specific amplification of genes less than 200 bp (Figure 1). Genes have introns. CDNA product size difference confirmed. no CDNA contamination with genomic DNA.

REAL-TIME PCR

Real-time PCR used the specific primers in Table 1 to amplify the Target Gene and House Kipping Gene. The reaction components were placed in the form of Cyber green Master Mix (12.5 µl), one µl each, one µl of CDNA sample, 8.5 µl of Deps Water, and a total volume of 25 µl in glass lid tubes. The real-time reaction was performed in 40 cycles on the model device (China Bioer) FQD 48A. Changes in gene expression compared to control samples were recorded using the formula 2-ΔΔCT. Three technical replications were given for each cDNA sample related to each sampling and used to analyze test data from Rest, Excel software.

RESULTS AND DISCUSSION

Drought stress is associated with an increase in the relative concentration of oxygen free radicals, especially hydrogen peroxide. Drought stress has negative effects on

physiological, biochemical, and morphological processes and ultimately affects knotting, nitrogen _xation, grain growth and yield, and soybean oil yield (Ashraf et al. 2011). Therefore, one of the economic strategies to increase grain yield and grain oil yield in producing products such as soybeans is the genetic modification of plants to tolerate drought stress (Shen et al. 2018). The results showed that the mean squares of tolerant and sensitive lines were significant at the level of 1% the mean squares of the environment at the level of 5% were significant, The interaction of the lines in the environment was No significant (Table 2). study of genes involved in drought stress reveals the possibility of analyzing the metabolic and physiological compatibility of plants under drought stress and study of the expression of genes involved in drought stress through biochemical, cellular, and molecular physiological processes determine how the plant responds to different environmental conditions (Thomashow 1999). The results showed that the increase in threshold changes for the CAT gene was 2.25, Changes in ASCP gene expression 5.45, GR gene expression 9.2 (figure 4). Studies show that the metabolic response in soybeans is maintained to drought stress (Guimarães-Dias et al. 2012). This means that during stress treatment, the average expression of the studied genes increases. The researchers noted that genes induced by drought stress reduced H2O2 and other reactive oxygen radicals (ROS) in tissues while reducing gas exchange in leaves. To neutralize the toxic and destructive effects of ROS, the enzymatic and non-enzymatic antioxidant defense system is activated in plant cells and the amount of oxygen free radicals is regulated by this system (Chun-miao et al. 2015) (de Paiva Rolla et al. 2014). The results of changes in the expression threshold of the studied genes showed an increase in the expression of the studied genes in stress treatment than normal conditions (Figure 2). Riechmann et al. 2000 in their study of physiological traits and changes in gene expression during the stress period intolerant and sensitive soybean Genotypes found that the tolerant genotype is better adapted due to the regulation of gene expression timing. Transcription agents are proteins that enhance or prevent gene expression by binding to specific DNA sequences (cis-acting) in the promoter region of target genes. The genes responsible for transcription factors make up many genomes in eukaryotes and plants (Riechmann et al. 2000). The results showed that the comparison of cycle threshold changes

of the studied genes showed that the CAT cycle threshold limit in drought-tolerant line in normal condition (GRO) treatment and drought-tolerant line in drought stress treatment (GSO) was higher than the threshold was the reference gene cycle threshold. This result indicates an increase in the expression of this gene in the cell before stress. As soon as intracellular changes occur, different Signal Transduction is initiated to convert physical stress into an appropriate biochemical response, each of which triggers the expression of a set of stress-responsive genes (Gill et al. 2016). The products of these genes not only act to protect cells from stress but also to regulate genes involved in stress response signaling (Kidokoro et al. 2015). Expression of CAT gene in drought-tolerant line (GRO) in normal irrigation treatment indicates high expression of this gene before stress treatment, However, the expression of CAT gene in stress treatment (GRS) was lower than the expression of this gene in normal conditions. abiotic stresses, such as drought and salinity stress, severely threaten plant growth and affect crop yield and quality (Li et al. 2017). The results showed that the expression threshold of (GR) gene expression was lower than the reference gene in both treatments (stress treatment and normal irrigation conditions) than the reference gene expression, but the resistant line had more expression in stress treatment and normal conditions treatment. This result showed that the expression of the Glutathione Reductase gene, like the expression of the Catalase gene in the resistant line before stress treatment, was higher than the sensitive line. The theory is also true that chaperone proteins produced during the plant growth cycle do not disappear to express the GR gene (Rajkowitsch et al. 2007). Plants use various defense mechanisms to counteract oxidative stress, including enzymatic and non-enzymatic mechanisms (WANG et al. 2018). The results of ASCP gene expression cycle threshold changes for drought-sensitive and resistant strains showed that drought-tolerant lines in drought stress treatment (G.S.O) had the greatest ASCP gene expression cycle threshold changes. The lowest ASCP expression cycle threshold changes were to drought stress-sensitive lines in drought stress treatment. The products of these genes can be classified into two groups based on their type of function. The first group includes proteins such as chaperones, water channel proteins, abundant late embryogenesis proteins (LEAs), osmolyte biosynthesis enzymes, detoxification enzymes, and membrane lipid-modifying enzymes involved in stress tolerance. The second group includes protein factors involved in regulating gene expression and signaling in response to abiotic stress, which includes kinases, enzymes involved in phospholipids metabolism, and Transcription Factor (Yamaguchi-Shinozaki and Shinozaki 2005). Changes in the expression threshold of the ASCP gene expression cycle indicate that the proteins produced by this gene have a high rate of expression before stress in cells. Other results have shown that in plants, the transcription factor can control the expression of many genes by binding specifically to the cis-acting element in the promoter region of target genes (Wang et al. 2016) (Shen et al. 2018).

Table 1- Gene description, primer sequence , and efficiency of selected primers for genes Glutathione Reductase (GR), (، Ascorbat peroxidase (Ascp) ، Catalase (CAT) Reference Gene FBOX Glyma12g05510.)

primer		Primer sequences	Number of nucleotides	Size bp	Tm	%GC	**Molecular weight g/mol**
Ascp	F	CCCCTCAGTGCCCAGTTTGG	20	155	63.06	65	**6044.9**
	R	TGCAGCTCTCCATCAAACAC	20		58.47	50	**6006**
CAT	F	GCCAACCACAGCCATGCCACT	20	188	63.3	61.9	**6305.1**
	R	AGGACCAAGCGACCAACAGGC	20		62.4	61.9	**6443.2**
GR	F	GCGCCCGAGTCACTCATCA	19	216	62.36	63.16	**5733.8**
	R	GCGACCCAACCAAATCACAGTCAA	24		64.06	50	**7268.8**
FBOX	F	CTAATGGCAATTGCAGCTCTC	21	93	53	47.6	**6381.2**
Glyma12g05510	R	AGATAGGGAAATGGTGCAGGT	21		56	47.6	**6599.3**

Table 2 - Sources of variation and mean squares of treatment for the studied genes

S.O.V	df	M.S
Line	1	57.722**
Environment	1	13.083*
line interaction in the environment	1	4.28 n.s
Error	8	37.713
Total	11	112.803

**,* و n.s Significant at the level of 1%, 5% and non-significant, respectively

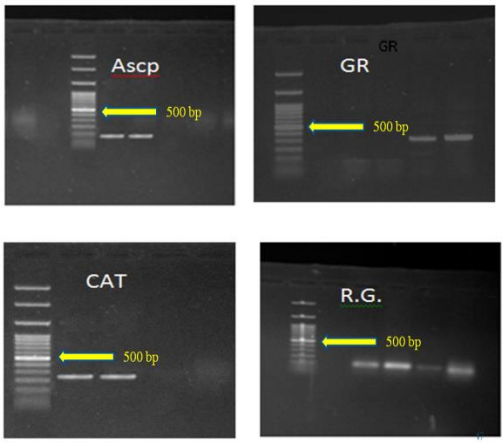

Figure 1- Confirmation of CDNA specific amplification for Catalase (CAT), Ascorbate peroxidase (Ascp),
Glutathione Reductase (GR) and R.G. reference genes: FBOX Glyma12g05510

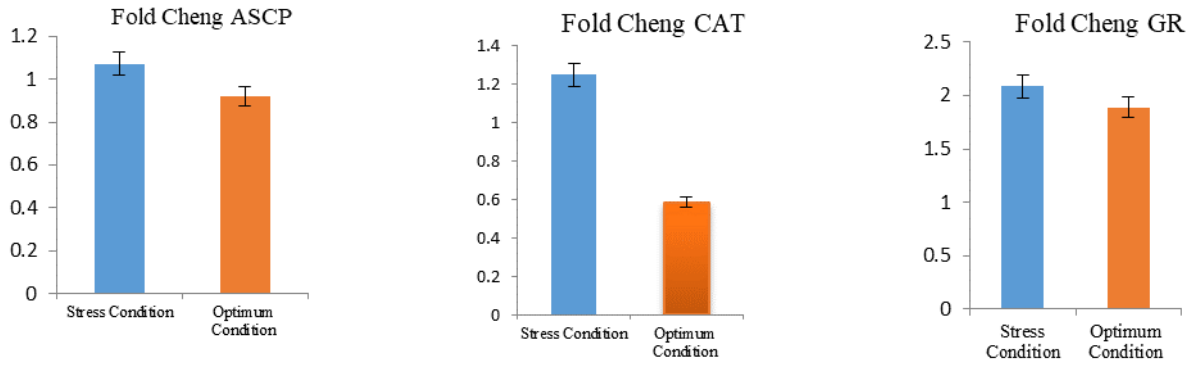

Figure 2- Cycle threshold changes in the studied genes for stress conditions and normal irrigation conditions

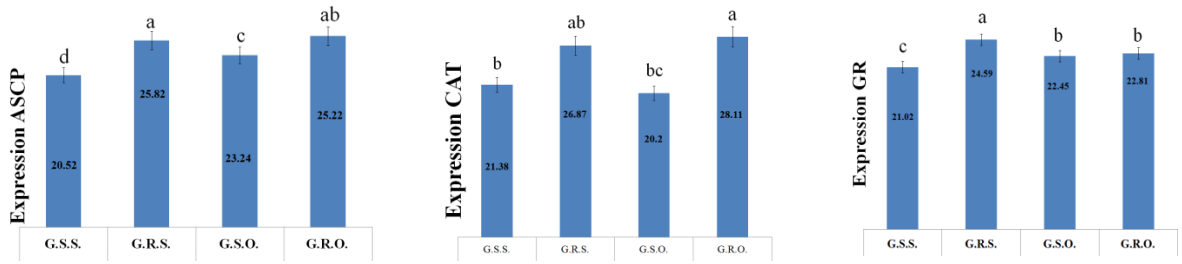

Figure 3- Cycle threshold changes for samples G.S.S: Line sensitive to drought stress in drought stress treatment. G.R.S: Drought stress-resistant line in drought stress treatment. G.S.O: Drought stress-sensitive line in normal irrigation treatment. G.R.O: Drought stress-resistant line in normal irrigation treatment

Catalase < Ascorbate peroxidase < Glutathione Reductase

Figure 4 - Comparison of expression of induced genes with drought stress intolerant and sensitive soybean lines

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