Comparison of Glutathione Reductase Gene Expression in Drought-Sensitive and Tolerant Soybean Lines Using Real-Time PCR

Mohammad Hassanvand, Shahab Khaghani, Mahdi Changizi, Masoud Gomarian, and Ezatollah Sedaghatfar

Department of Genetic and Plant Breeding, Arak Branch, Islamic Azad University, Arak, Iran
Department of Plant Protection, Arak Branch, Islamic Azad University, Arak, Iran
* e-mail: shahab.khaghani@gmail.com
Received March 17, 2022; revised July 13, 2022; accepted July 13, 2022

Abstract—Global climate change and associated adverse abiotic stress conditions, such as drought, salinity, heavy metals, waterlogging, extreme temperatures, oxygen deprivation, etc., greatly influence plant growth and development, ultimately affecting crop yield and quality, as well as agricultural sustainability in general. This study provides new insights into the analysis of the function of soybean genes in abiotic stress. Drought is one of the significant constraints that limit agricultural productivity. Some factors, including climate changes and acreage expansion, indicate the need for developing drought-tolerant Genotypes.

Materials and methods: The study of the expression of Glutathione Reductase (GR) gene in soybean drought-tolerant and sensitive cultivars using real-time PCR. Seeds from (drought-sensitive) and (drought-tolerant) lines were planted under specific temperature conditions for drought stress treatment, in the research greenhouse of Islamic Azad University of Arak, Iran. Changes in gene expression compared to reference genes 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 MINITAB16 software.

Results: The results showed that the threshold expression of gene expression (Glutathione) in the Pyramid line had the highest expression of drought resistance and the lowest expression of the Glutathione Reductase gene belonging to the Will line. The theory is also true that chaperone proteins produced during the plant growth cycle are not destroyed to express the Glutathione Reductase gene. The expression cycle of the Glutathione Reductase gene shows that the proteins produced by this gene have a high rate of expression and increase in cell drought stress. This gene expression continues until the pressure ends. The results showed that lines and cultivars with a weak expression against drought stress could have a high expression at the beginning of drought stress but a decrease in gene expression rate during stress. Drought stress-sensitive lines have a decreasing expression in the middle and end of stress during the stress period.

Keywords: gene expression, glutathione Reductase, sensitive line, resistant line, real-time PCR
DOI: 10.3103/S1068367422060118

INTRODUCTION

Global climate change and associated adverse abiotic stress conditions, such as drought, salinity, heavy metals, waterlogging, extreme temperatures, oxygen deprivation, etc., greatly influence plant growth and development, ultimately affecting crop yield and quality, as well as agricultural sustainability in general. Plant cells produce oxygen radicals and their derivatives, so-called reactive oxygen species (ROS), during various processes associated with abiotic stress.

Moreover, the generation of ROS is a fundamental process in higher plants and employs to transmit cellular signaling information in response to changing environmental conditions. One of the most important consequences of abiotic stress is the disturbance of the equilibrium between the generation of ROS and antioxidant defense systems triggering the excessive accumulation of ROS and inducing oxidative stress in plants. Notably, the balance between the detoxification and generation of ROS is maintained by both enzymatic and no enzymatic antioxidant defense systems under extreme environmental stresses.

Although this field of research has attracted tremendous interest, it largely remains unexplored, and our understanding of ROS signaling remains poorly understood [1]. During stress conditions, over generation of ROS demolishes the equilibrium and causes cellular damage, leading to programmed cell death (PCD) and decreasing plant productivity [2]. Drought is an adverse environmental factor affecting crop
Table 1. Drought stress lines for determination of drought stress resistance using Glutathione Reductase gene expression

| Line | Name        |
|------|-------------|
| 1    | Pace        |
| 2    | Dare        |
| 3    | Odell       |
| 4    | Prohio      |
| 5    | S39-99      |
| 6    | crowford    |
| 7    | Douglas     |
| 8    | Tiffin      |
| 9    | Pella       |
| 10   | PI 475822 A|
| 11   | Columbus    |
| 12   | Will        |
| 13   | Williams    |
| 14   | Amcor 89    |
| 15   | Xiao Wuyie  |
| 16   | Pyramid     |

growth, development, and yield. Promising genotypes and genes with improved tolerance to drought are probably effective ways to alleviate the situation [3]. Drought is considered a significant threat to soybean production worldwide [4].

Drought is a severe but infrequent stressor affecting soybean production [5]. Abiotic stresses severely affect the growth, development, and ultimately yield of the plant, which results in heavy economic losses and food crises. Oxidative stress, associated with almost all abiotic stresses, is due to the overproduction of toxic reactive oxygen species (ROS), including superoxide ions, hydrogen peroxide, and hydroxyl radicals. Plants combat oxidative stress via enzymatic and non-enzymatic machinery [6].

Drought stress reduced the chlorophyll content and relative water content in the soybean leaves and increased the osmolyte contents, antioxidant potential, and peroxidation of the membrane lipids [7]. GR is an increase in the activity of plant stress markers in the plant [8]. By measuring glutathione Reductase, the degree of stress sensitivity in the plant can be measured. glutathione, Reductase is responsible for maintaining the supply of reduced glutathione; one of the most abundant reducing thiols in the majority of cells. In its reduced form, glutathione plays a key role in the cellular control of reactive oxygen species.

Reactive oxygen species act as intracellular and extracellular signaling molecules and complex crosstalk between levels of reactive oxygen species, levels of oxidized and reduced glutathione and other thiols, and antioxidant enzymes such as glutathione Reductase determine the most suitable conditions for redox control within a cell; In general, insects and kinetoplastids (a group of protozoa, including Plasmodia and Trypanosoma) do not express glutathione Reductase or Glutathione biosynthetic enzymes [9]. The evidence indicates an essential role of Glutathione, Reductase, and Glutathione Reductase as components of a peroxide-scavenging mechanism in the soybean cell body [9].

MATERIALS AND METHODS

Drought-resistant and drought-sensitive seeds were obtained from Karaj Seed Breeding Research Institute, which are imported cultivars (Table 1). Seeds were sown in drought stress treatment at temperature (30 ± 2°C) and 16 h of light (20 ± 2°C) and 8 h of darkness, in the research greenhouse of Islamic Azad University, Arak city, Markazi province.

Leaf was up to 7 days (prolonged stress). To examine the expression of drought stress genes on day 5, leaf sampling was performed by observing the tubularity of the leaves (simple leaves are open enough). 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) PR891620-EX6031. Using Nanodrop to determine the concentration (NanoDrop 1000 spectrometer), the absorbance of each sample was recorded at 230, 260, and 280 nm.

CDNA (Sinaclon First Strand DNA Synthesis Kit-50T) 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. For 16 samples the material was mixed and, an additional unit (test unit) was added to the extraction solution (34 μL). 0.5 μL of DEPC water was added to each sample and one RNase inhibitor unit was added to the microtube (total 8.5 μL and 8 h of darkness, in the research greenhouse of Islamic Azad University, Arak city, Markazi province.

Leaf was up to 7 days (prolonged stress). To examine the expression of drought stress genes on day 5, leaf sampling was performed by observing the tubularity of the leaves (simple leaves are open enough). 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) PR891620-EX6031. Using Nanodrop to determine the concentration (NanoDrop 1000 spectrometer), the absorbance of each sample was recorded at 230, 260, and 280 nm.

CDNA (Sinaclon First Strand DNA Synthesis Kit-50T) 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. For 16 samples the material was mixed and, an additional unit (test unit) was added to the extraction solution (34 μL in total). 0.5 μL of DEPC water was added to the tube for 16 samples, and another unit. The tube containing the CDNA solution was placed in Ban-marry for 60 min at +42°C and stopped the reaction at 85°C for 5 min. Samples were stored at −20°C. To design the primer the gene sequences of 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 2). The primers were synthesized by SinaClone Company. PCR and electrophoresis were performed to ensure the specific performance of the primers. PCR prod-
ucts were observed on 1% agarose gel to provide specific amplification of genes less than 200 bp (Fig. 1).

Genes have introns. CDNA product size difference confirmed. CDNA was without contamination with genomic DNA. 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 Dmp 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 reference genes 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 analyse test data from MINITAB16, Excel software.

### DISCUSSION

Real-time PCR can quantify CDNA by amplifying energy using a threshold cycle that continues to resist. Real-time PCR programs detect different types of stresses in resistance-specific resistance-specific sensitivity and specificity programs. In addition, real-time PCR performance requires test protocol performance by measuring sensitivity, specificity, accuracy, and reproducibility. Approved real-time PCR is an easy, fast, and accurate way to monitor the results of diagnosis and treatment in a stressful environment [10]. Real-time PCR can analyses hundreds of samples in a day [11]. In environments where water is limited, genetic improvement of a crop for drought resistance is an economically attractive option [13].

However, despite the extensive resources committed to soybean breeding, progress in improving drought resistance has been slow for several reasons.

---

**Table 2. Primer characteristics used and reference gene**

| Primer name     | Primer sequence         | N.N | Primer size | T.m  | %GC | M.W, g/mol |
|----------------|-------------------------|-----|-------------|------|-----|------------|
| Glutathione    | GCGCCCGAGTCACTCATCA     | 19  | 216         | 62.36| 63.16| 5733.8     |
| Reductase      | GCGACCCAACCAAATCAGCTCA  | 24  | 64.06       | 50   |     | 7268.8     |
| FBOX           | CTTATGGCAATTTCAGCTTC    | 21  | 93          | 53   | 47.6 | 6381.2     |
| Glyma12g05510  | AGATAGGGAAATGGTCAGGT    | 21  | 56          | 47.6 |     | 6599.3     |

**Fig. 1.** Confirmation of CDNA-specific amplification for Glutathione Reductase (GR) and R.G. reference genes: FBOX Glyma12g05510.
Identifying lines with the highest yield potential under optimum moisture conditions is an important selection criterion in soybean. Conversely, evaluating lines from low-yielding environments under drought conditions is often not considered, because small yield differences among lines do not separate high-yielding genotypes from low-yielding genotypes [14]. Historically, the emphasis in soybean breeding was upon resistance to biotic stress rather than abiotic stress such as drought, due to the complexity of trait evaluation. This, unfortunately, resulted in a narrow genetic base for initiating drought resistance breeding programs [15].

RESULTS

The results of the analysis of variance tables of CT1s obtained from the analysis of real-time PCR data showed a significant difference at the level of 1% between the studied lines (Table 3).

The results of comparing the threshold cycles of drought-sensitive and tolerant lines in soybean showed that the pyramid line had the highest gene expression and showed a significant difference at the level of 1% with other lines. This reported that the pyramid line is also resistant to living stresses [12]. Williams, Columbus, and Douglas lines did not differ significantly in the expression of drought resistance gene; these lines had the highest expression of GR gene to drought stress after the pyramid line, called semi-drought resistance lines. The lowest expression of the GR gene and the most sensitive to drought stress will line. Although tiffin, Xiaowuyie, and Amcor89 lines did not differ significantly in GR gene expression, these lines showed high sensitivity to drought stress (Fig. 2).

In the study of drought stress resistance and sensitivity using cluster analysis and using Euclidean distances, it can be concluded that Pace and will lines were placed in a separate cluster. The pyramid line, which had the highest expression of the GR gene in drought resistance expression, was placed in a cluster with s39-99, Columbus, and PI475822a lines. Lines in a cluster with the pyramid line have the genetic potential to increase drought stress resistance. The Amcor89 and Xiaowuyie lines were in a separate cluster, and the will, Douglas, and Crawford lines were in a separate cluster. The Euclidean distance diagram (Fig. 3) for future crossings in improving and increasing the expression of the GR gene is forward-looking, showing the best location for subsequent crossings by the dashed line.

The results of the plot matrix for comparing the first and second threshold cycles (CT1 and CT2) according to the formula $2^{-\Delta \Delta CT}$ showed that the most significant difference in the first and second threshold cycles is related to the Dare line. On the other hand, the high expression results of the Pyramid line gene start in the threshold 22 cycles, while in the threshold cycle, the Dare line starts in the 30th cycle. A Com-

---

**Table 3. Analysis of variance for CTs obtained from PCR real-time analysis**

| S.O.V  | DF | SS   | MS    |
|--------|----|------|-------|
| Line   | 15 | 461.122 | 30.741* |
| Error  | 32 | 58.627  |       |
| Total  | 47 |        |       |

* Significant 1%.

---

Fig. 2. Comparison of Fold Chang of lines in Glutathione Reductase gene expression.
Comparison of GR gene expression in comparing the first and second threshold cycles (CT1 and CT2) showed that GR gene expression was strongly reduced in Crowford and Will lines.

In comparison, the expression of the GR gene in the CT1 and CT3 threshold cycles of the Douglas line was decreasing, but in the CT3 threshold cycle, this gene expression was increasing. The results of comparing the GR gene expression threshold cycle in the second threshold cycle showed that they would line acted out of expression, meaning that due to the lack of GR gene expression in the will line, the real-time PCR device could not calculate this amount of gene expression (Fig. 4).

Comparing the second and third threshold cycles in GR gene expression in the 22-line will cycle again showed the lowest gene expression. The lines that are expressed earlier are indeed the lines that feel the drought stress sooner and have to release their genes and enzymes sooner, but this gene expression and stress identification must continue while lines like tiffin, Amcore, and Xiaowuyie had earlier gene expression, but this gene expression is not continuous, so it is observed in stress-sensitive cells.

Clearance of free radicals in cells by resistance genes clearing them is acceptable when gene expression continues until stress is relieved. Lines that express their stress resistance genes earlier are not the reason for their stable resistance, but a line should have both fast and high gene expression and continuous expression of that gene.

Fig. 3. Dendrogram for hybridization that is associated with increased Glutathione Reductase levels.

Fig. 4. Comparison of the threshold cycle of the first cycle data with the second cycle.
Resistance in lines is acceptable when the expression of amino acids and resistance-building proteins is done to the end of stress relief. Accelerated gene expression is not a sufficient reason for resistance to drought stress. Sometimes some lines have a high rate of gene expression, but it is observed that the cell does not show sufficient gene expression, and the line will not be sensitive to stress, or the cell will lose its vital activity due to stress and will disappear. Compared to the first and third threshold cycles (Fig. 5), the odel line had the fastest expression in cycle 20, but the result of continued expression of the GR gene was again decreasing. As mentioned, a line can be called resistant that continues to express its stability against drought stress. The lines that have their threshold changes compared in Fig. 6 and are outside the standard line of incremental gene expression are the lines that have decreased threshold changes. The will line had the lowest expression with a decreasing trend compared to the second and third thresholds.

Fig. 5. Comparison of the threshold cycle of the first cycle data with the third cycle.

Fig. 6. Comparison of the threshold cycle of the second cycle data with the third cycle.
AUTHOR CONTRIBUTION

All authors contributed to the study’s conception and design. Material preparation, data collection, and analysis were performed by Mohammad Hassanvand. The first draft of the manuscript was written by Mohammad Hassanvand and I commented on previous versions of the manuscript. I read and approved the final manuscript.

TAXONOMY

Conceptualization: Mohammad Hassanvand; Methodology: Shahab Khaghani; Formal analysis and investigation: Mohammad Hassanvand and Ezatollah Sedaghatfar; Writing—original draft preparation: Mohammad Hassanvand; Writing—review and editing: Mahdi Changizi and Masoud Gomarian; Resources: Mohammad Hassanvand; Supervision: Shahab Khaghani.

FUNDING INFO

This article has been prepared from a research project affiliated with the Agricultural Education and Extension Research Organization of Iran under the identification no. 18785758 of the Seed and Seedling Breeding Institute. Mohammad Hassanvand is the executor of the research project. The data has been provided to the researcher with the permission of this organization and the costs related to the project have been paid by the research organization. The organization authorized the executor to use the data.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Hasanuzzaman, M., Bhuyan, M.H.M.B., Zulfiqar, F., Raza, A., Mohsin, S.M., Al Mahmud, J., et al., Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator, Antioxidants, 2020, vol. 9, no. 8, p. 681.
2. Raja, V., Majeed, U., Kang, H., Andrabi, K.I., and John, R., Abiotic stress: Interplay between ROS, hormones and MAPKs, Environ. Exp. Bot., 2017, vol. 137, pp. 142–157.
3. Chen, L., Fang, Y., Li, X., Zeng, K., Chen, H., Zhang, H., et al., Identification of soybean drought-tolerant genotypes and loci correlated with agronomic traits contrib-
utes new candidate genes for breeding, Plant Mol. Biol., 2020, vol. 102, nos. 1–2, pp. 109–122.
4. Kunert, K.J., Vorster, B.J., Fenta, B.A., Kibido, T., Dionisio, G., and Foyer, C.H., Drought stress responses in soybean roots and nodules, Front. Plant Sci., 2016, vol. 7, p. 105.
5. Licht, M.A., Wright, D., and Lenssen, A.W., Soybean response to drought, Agric. Environ. Ext. Publ., 2013, vol. 190, pp. 1–3.
6. Youssuf, P.Y., Rehman Hakeem, K.U., Chandra, R., and Ahmad, P., Role of glutathione reductase in plant abiotic stress, in Abiotic Stress Responses in Plants: Metabolism, Productivity and Sustainability, New York: Springer-Verlag, 2012, pp. 149–158.
7. Dong, S., Jiang, Y., Dong, Y., Wang, L., Wang, W., Ma, Z., et al., A study on soybean responses to drought stress and rehydration, Saudi J. Biol. Sci., 2019, vol. 26, no. 8, pp. 2006–2017.
8. Trivedi, G., Patel, P., and Saraf, M., Synergistic effect of endophytic selenobacteria on biofortification and growth of Glycine max under drought stress, South Afr. J. Bot., 2020, vol. 134, pp. 27–35.
9. Couto, N., Wood, J., and Barber, J., The role of glutathione reductase and related enzymes on cellular redox homoeostasis network, Free Radical Biol. Med., 2016, vol. 95, pp. 27–42.
10. Hwang, K.A., Ahn, J.H., and Nam, J.H., Diagnosis of viral infection using real-time polymerase chain reaction, J. Bacteriol. Virol., 2018, vol. 48, pp. 1–13.
11. Li, Z., Hansen, J.L., Liu, Y., Zemetra, R.S., and Berger, P.H., Using real-time PCR to determine transgene copy number in wheat, Plant Mol. Biol. Rep., 2004, vol 22, no. 2, pp. 179–188.
12. Ortega, M.A., All, J.N., Boerma, H.R., and Parrott, W.A., Pyramids of QTLs enhance host–plant resistance and Bt-mediated resistance to leaf-chewing insects in soybean, Theor. Appl. Genet., 2016, vol. 129, no. 4, pp. 703–715.
13. Blum, A., Drought tolerance—is it a complex trait?, in Field Screening for Drought Tolerance in Crop Plants with Special Emphases on Rice, Patancheru: Int. Crop Res. Inst. Semi-Arid Trop., 2002, pp. 17–22.
14. Carter, Jr. T.E., Recent advances in breeding for drought and aluminum resistance in soybean, Proceedings of World Soybean Research Conference, Chicago, 1999, pp. 106–125.
15. Manavalan, L.P., Guttikonda, S.K., Phan Tran, L.S., and Nguyen, H.T., Physiological and molecular approaches to improve drought resistance in soybean, Plant Cell Physiol., 2009, vol. 50, pp. 1260–1276.