



*Research article*

## Evaluation of proline, soluble sugar and ABA content in soybean *Glycine max* (L.) under drought stress memory

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**Abstract:** Drought stress memory in plant can alter their physiological, biochemical and molecular to a subsequent stress. An experiment was conducted to determine biochemical parameters of soybean seedlings under drought stress memory. 14-days-old soybean seedlings were subjected to three consecutive water deficit phases (D1, D2, D3), each phase recovered by re-watering (R1, R2, R3), and control plant watering daily (R0). Leave of seedlings from these phases were collected and analyzed. Significantly increasing contents of soluble sugar and proline observed at the first drought stress. After the first re-water and continuous stress phases, these indicator contents decreased and maintained at a relatively stable level. Expression level of two memory genes encoded to transcription factor (NAC09 and NAC109) and one gene encoded enzyme  $\Delta$ 1-pyrroline-5-carboxylate synthetase (P5CS1) were increased in the first stress and decreased in third stress. Our results demonstrate that changing of biochemical parameters of soybean seedlings can be seen as the strong indications of “drought stress memory”. This result may serve as a reference platform to study advanced researches at molecular and genetic levels.

**Keywords:** ABA; proline; transcription factor; soybean; drought stress memory

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## 1. Introduction

Climate change threatens stable crop yields, will likely require changes in agricultural practices in response to increased consumption. Drought is one of the most severe problems to plants, affecting plants from the molecular, cell and organ to the whole body [1,2]. When plants live in a dehydrate environment, the tissue becomes soft and wilting, and directly affects the metabolic and morphological activities of plants, then leading to decrease the growth and development. Adjustment of osmotic pressure is a way to cope with the drought in plants. The synthesis of solutes such as proline and carbohydrate molecules which help plant prevent dehydration and play an important role in maintaining cellular strength. Carbohydrate molecules provide growth activities under normal conditions and used for the synthesis of osmotic regulators [3]. Production and accumulation of proline content in plant under to various oxidative stresses enhances antioxidant defense system [4]. The proline biosynthesis is essentially regulated at the transcription level of  $\Delta$ 1-pyrroline-5-carboxylate synthetase 1 (P5CS1) [4]. Many reports indicated that increasing expression level of P5CS1 gene enhanced proline content in plants under stress tolerance [5]. Székely (2008) demonstrated that proline accumulation in P5CS1 knock-out mutants was decreased and increased sensitivity to osmotic stress, thus P5CS1 is necessary and sufficient to accumulate proline [6].

Phytohormone abscisic acid (ABA) level is rapidly induced and related to regulate stomatal closure in plants under drought stress. Increasing ABA level can active physiological responses and signaling transduction [7]. In addition, articles indicated that drought stress-related genes encode transcription factor in plants. TFs act as switches that affect a variety of genes respond to abiotic stress. Transcription factor (TFs) act as switches that affect a variety of genes in plant respond to abiotic stress. Le et al. (2011) detected the increase expression of 58/152 TFs NAC genes in soybean under drought stress [8]. Gene encoded transcription factor (NAC016) that related to ABA-mediated pathway have function in the drought response in *Arabidopsis thaliana* [9]. “Stress memory” refers to many changes in the physiological, proteomic, and transcriptional levels and also in the epigenetic mechanism in plants). Recent researches have focused on exploring the mechanisms of stress training and memory of various plant species responding to drought conditions [10,11]. Avaramova et al. (2015) suggest that drought or other abiotic stresses are encountered throughout the plant life cycle [12]. In order to survive the repeated stresses, plants must respond to the next stresses other than the initial response to stress, which is the memory of stress in plants. When plants are exposed to adverse conditions at the first time, it is likely that the plant will increase its resistance to the later times at higher levels. Many reports have verified that plants can respond quickly or strongly, leading to increased resistance to biotic and abiotic factors from the environment [13]. Results of researchers at International Center for Potato Research (Peru) demonstrated that seed tubers produced by plants grown under normal water (non-primed tubers) were similar ones exposed to long term stress memory (primed tubers) [14]. Sani et al. (2013) studied the memory mechanism of *Arabidopsis* through chromatin marks such as histone modification in both drought tolerant and non-drought tolerant plants [15]. The results show that plant growth and development are tolerated in the same way as untreated plants.

Although, recently, memory mechanism related to response to environmental stress has become the focus of plant research. But not many studies have been studied on the mechanisms of memory drought stress in *Glycine max* (L.) soybean plant. Therefore, this article has investigated that how the metabolism-related substances such as proline, sugar, ABA and transcription factors in soybean

under drought stress memory will be change. The results of this paper will provide basic knowledge about the memory ability of soybean plants under drought conditions.

## 2. Materials and method

### 2.1. Plant material, growth and stress conditions

Soybean seeds (*Glycine max* DT2008) supplied by Agricultural Genetics Institute, Vietnamese Academy of Agricultural Science, Vietnam and were used for all experiments in this study.

Seedlings were grown directly in plastic pots (6 seeds/pot) in greenhouse conditions (30 °C temperature, photoperiod of 12h/12h, illumination intensity 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and 60% relative humidity) and well-watered daily. Plants were divided into control and stress treatment, three replicates per treatment. 14-days-old seedlings were used as the first drought stress treatment during three days (D1), and fully re-watered at the 4<sup>th</sup> day (R1) during 3 days. Drought and re-water treatments were repeated for 2 rounds continuously (D2, R2, D3, R3 respectively). Control seedlings were watered daily throughout the experimental periods (R0). Leaf samples from R0, D1, R1, D2, R2, D3, R3 seedlings were collected and stored in -70 °C.

### 2.2. Total soluble sugar and proline content measurement

Total soluble sugars and proline contents were determined using spectrophotometer [16]. The total soluble sugar concentration was calculated by comparison with the standard curve using glucose at 625 nm wavelength. The total proline concentration was calculated by comparison with the standard curve using L-proline at 520 nm wavelength. Three replicates were prepared.

### 2.3. ABA content measurement

Leaf tissues were ground under liquid nitrogen. 200 mg fine power was extracted in 10 ml 80% (v/v) methanol medium containing 1 mM butylated hydroxytoluence. The extract was incubated at 4 °C overnight in darkness using small shaker, and then centrifuged at 12000 rpm for 20 min at 4 °C. The supernatant was filtered using Chromosep C18 columns. ABA fractions eluted with 10 ml 100% (v/v) methanol, dried by N<sub>2</sub> and dissolved in 1ml TBS (phosphate buffer saline and Tween 20). ABA content was analyzed by the Phytodetek ABA test kit (Agdia, Elkhart, IN, USA) following the manufacturer's instruction. Color absorbance was measured at 405 nm using a microplate reader (PowerWave XS, BioTek Instruments Inc.).

### 2.4. Isolation of RNA purification, cDNA synthesis and qRT-PCR

Leaf samples under control and drought stress memory were harvested, and ground under liquid nitrogen. Total RNA was extracted using of the Gene All Ribospin Plant Mini Kit (GeneAll Biotech, Korea), adding DNase I to remove DNA in this step. Concentrations of total RNA samples were quantified using a Nanodrop ND 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA samples (> 1  $\mu\text{g}/\mu\text{l}$ ) with high purity (OD<sub>260/280</sub> and OD<sub>260/230</sub> > 1.8) used for cDNA and RT-PCR. cDNA was performed from 1  $\mu\text{g}$  of total RNA from each sample with a SuperScript II Reverse Transcriptase and oligo (dT<sub>20</sub>) by incubation at 46 °C for 20 min and stopped at 95 °C for 1 min.

The primer sequences of selected genes for qRT-PCR are described in Table 1. These primer oligomers were designed using NCBI website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). qRT-PCR reaction was carried out in a 20  $\mu$ l mixture with 1  $\mu$ l of diluted cDNA template and SYBR Premix Ex TaqII (Takara, Japan). Actin gene was used as an internal control to ensure that equal amounts of cDNA in all the reactions. All experiments were repeated at least 3 times for both Actin and selected genes.

**Table 1.** List of primer sequences.

Gene name	Gene ID	Primer sequence
NAC109	Glyma.14G152700	ACATATCGCGGTTCCCATAA CGGTTTCGGTTTACCAACTG
NAC019	Glyma.04G212000	CTCTCATTCATGCCATCCT TGACACCTGTGTCCTTCCAA
P5CS1	Glyma.18G034300	CGAACTGAGCTTGCAGAGGGGC TCGCTTAGCCTCCTTGCTCC

### 2.5. Statistical analyses

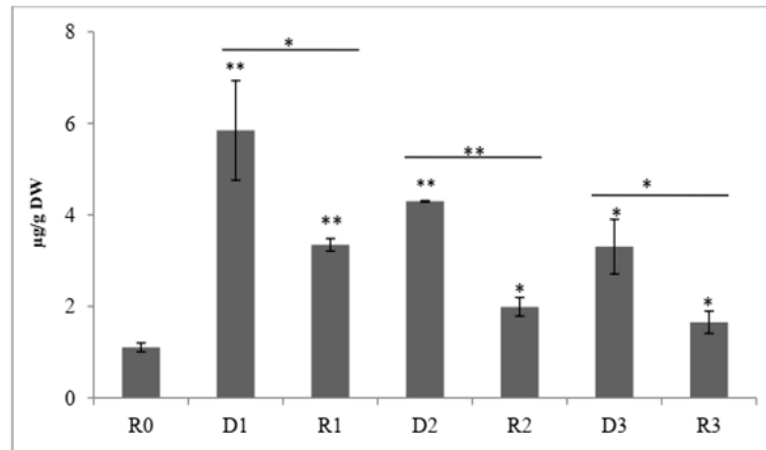
The statistical significance of ABA, soluble sugar and proline contents of were evaluated through t-test with  $p < 0.05$ , between well-water (control) plants and temporarily non-irrigated (stress and recovery) plants.

## 3. Results

### 3.1. Proline and sugar contents

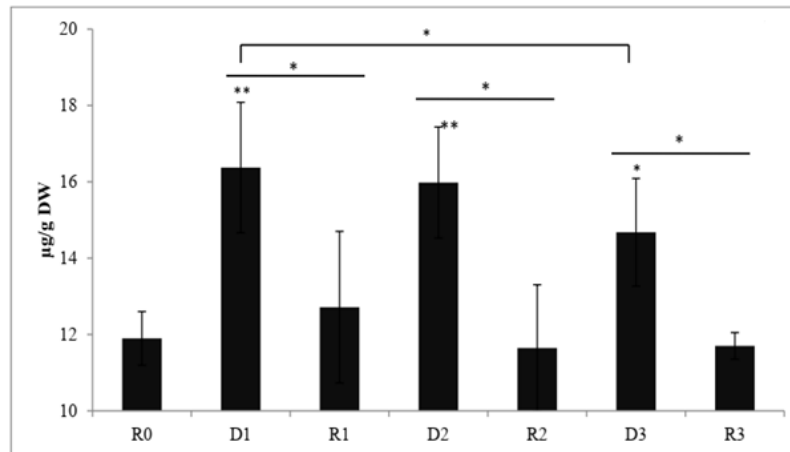
Metabolic alterations include accumulation of proline and sugar induced by drought stress in plants. To check if proline and sugar are involved in soybean drought memory response, we measured the proline and sugar concentrations from R0 to R3 by a spectrophotometer. Modification of leaf proline and sugar concentration to water deficit and re-water are show in Figures 1 and 2, respectively.

Proline content of seedlings response to drought stress changed significantly compared to control. Proline contents were 5.29-fold higher in D1 plants than in R0 plants (Figure 1). After the second and third re-watering phases, proline level decreased to less than 1/3 of those in D1 plants. At the same time, we also checked the difference among groups D1-R1, D2-R2 and D3-R3. The results showed that there are significant differences in these groups. The difference changes of proline contents after drought stress periods indicated that soybean drought stress memory responses possibly maintaining homeostasis of proline levels.



**Figure 1.** Influence of water stress and re-watering on the proline concentration of soybean.

To understand whether soluble sugar accumulation is memorable during repeated drought stress, the free soluble sugar amount in 14-days-old soybean seedlings was examined with water deficit treatment and recover conditions. The sugar content strongly increased after the first drought stress (D1) after the long-term drought stress, and diminished with re-water condition (R1). We also compared the difference among groups D1-R1, D2-R2 and D3-R3. The results showed that there are significant differences in these groups (Figure 2), suggesting that sugar is also involved in soybean drought memory response.

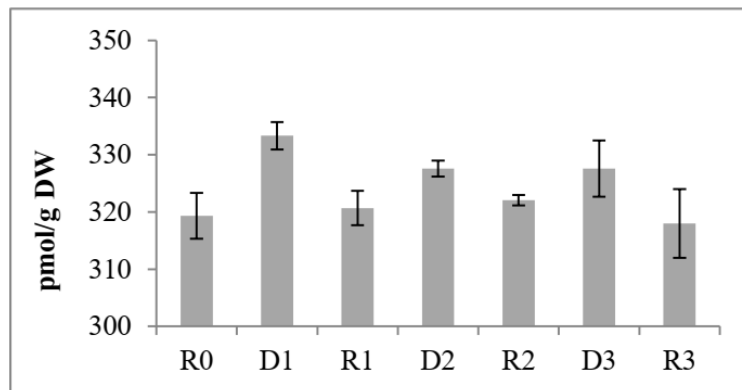


**Figure 2.** Soluble sugar concentration in the leaf tissues of soybean subjected to water deficit conditions.

### 3.2. ABA content in drought stressed soybean

Many studies demonstrated that the metabolic alterations induced by drought stress in plants such as hormone ABA. To evaluate if ABA are related to soybean drought memory response, we quantified ABA concentrations at several time points from R0 to R3 by ELISA assay. After long-term drought stress durations, the level of endogenous ABA in soybean leaf tissues under water deficit and re-water conditions changed significantly (Figure 3). We found that, the ABA content

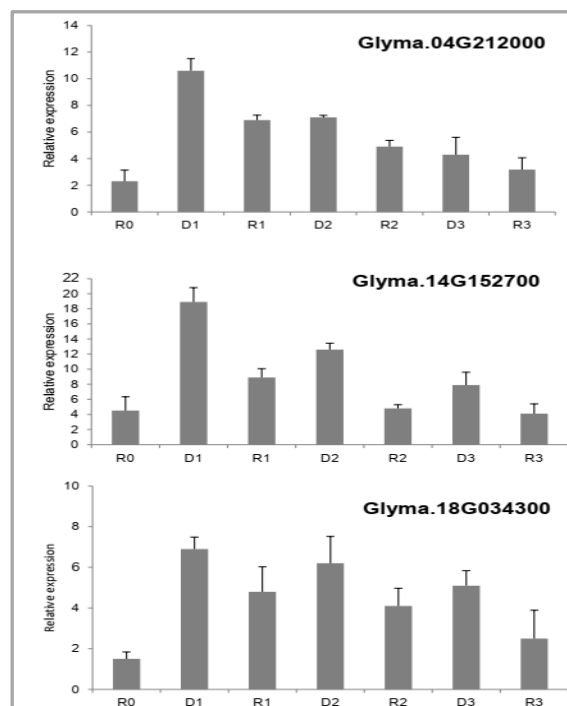
increased at the first drought stress, while a progressively decrease level was recorded in next stresses. The endogenous ABA level reduced after re-watering treatments (R3) with a value similar to that of control plants.



**Figure 3.** Effect of drought stress on endogenous ABA levels in the leaf tissues of soybean.

### 3.3. Expression of the water deficit induced genes

Two genes encoded NAC TFs (NAC19 Glyma.04g212000 and NAC109 Glyma.14g152700) and one gene encoded enzyme  $\Delta$ 1-pyrroline-5-carboxylate synthetase 1 (P5CS1, Glyma.18g034300) were determined relative expression. Results indicated that the expression of two genes related to NAC-TFs and P5CS1 increased dramatically and reached the highest value at the first drought stress (D1), and decreased in R1 and subsequent times (Figure 4).



**Figure 4.** The expression of two genes encode to NAC transcription factor and P5CS1 (respectively from top to bottom) in the leaf tissues of soybean.

#### 4. Discussion

Drought-stressed plants have been indicated that accumulate organic osmolytes such as sugars and proline that are known to contribute to tolerance in plants subjected to water-deficit conditions [17]. Proline plays a vital role in maintaining optimal growth in plant under biotic stresses [4]. The significantly enhanced proline content in soybean leaf at the first drought stress is a response characteristic of plants like as osmotic adjustor under abiotic stresses. Proline promotes higher resistance in plant cells under adverse environmental conditions, as well as acting a molecular chaperon to stabilize protein structure. According to Meena et al. (2019), the increase in the content of proline in drought stress—subjected plants may provide high energy to promote plant growth in water deficit condition [4]. Similar results were observed in sugar beet plants subjected to three consecutive water deficit phases [18]. The soluble sugar amount was immediate increasing in soybean seedlings under the first drought stress. In the recovery phase, values of soluble sugar reached those of control plants. Accumulation of soluble sugar in plant cells subjected to drought stress is responsible for osmotic adjustment. Besides, soluble sugar plays an important role in osmotic adjustment in plants. The accumulation of sugars in drought stressed plants is controlled by several mechanisms affecting soluble sugar formation and transfer in leaves [19]. Similar results in the increasing accumulation in the total soluble sugar were demonstrated by Lobato et al. (2008) soybean plants under drought stress [20]. The results of this study showed that an increase in first drought stress led to increase leaf soluble sugar concentration compare to the re-water treatments. Additional, the soluble sugar contents were found to increase in alfalfa seedlings subjected to drought stress. The higher soluble sugars level in water stressed plants plant leaves (D1, D2 and D3) than other treatment plants and indices recover soon after re-watering (R1, R2 and R3), which suggested to associated with more drought resistance of the plants. Progressively increase levels of soluble sugar and proline in plants under water deficit improved the resistance of the plants under water deficit condition [20,21]. Interestingly, both of proline and soluble sugar amounts declined progressively in plants under re-water conditions, indicating rapid metabolism of these metabolites following removal of the stress. Similar results demonstrated that proline accumulation in rice under drought memory stress [22].

Plant growth hormone as abscisic acid (ABA) plays a vital role in plant growth and development, and their responses are significant signal in understanding acclimation mechanism of plants. Studies indicated that an increase in ABA amount was due to osmotic stress in plants subjected abiotic stresses, such as drought stress, salinity and cold [23,24]. The water deficit at the first stress triggered a significant enhance in endogenous ABA amount in soybean leaf tissues, and reduced at every re-watering treatment and remained at a constant level with R0 after three rounds of treatment. Our results were similar to published reports [22,25,26]. The maintaining homeostasis of ABA level might relate to the memory response to drought stress in soybean. According to Li et al. (2019), transcriptomic analyses showed that genes encode to ABA-related pathway indeed participate in short-term drought memory in rice under drought stress [22].

Many studies indicated that NAC TF's are up-regulated by abiotic stresses as well as ABA, salinity and drought. Ding et al. (2014) discovered number of memory genes related to NAC family genes in maize and *Arabidopsis* [27]. ANAC019 involved in ABA-mediated signaling, and playing role in regulation of defense response under biotic stress of plant [28]. Expression of transcription factor ANAC019 is demonstrated that related to JA and ABA pathways under drought stress

regulation [29]. In this study, increased expression of P5CS1 was also determined. Changes in expression levels of P5CS1 have been reported in drought memory research in rice and in tobacco [22,30]. These results suggest that proline is definitely associated with drought memory response of soybean.

## 5. Conclusion

Our study indicates that the parameters to changing assess the biochemical of soybean seedlings and provide strong indications of “drought stress memory”. The maintaining homeostasis of proline, total sugar and ABA levels may be important in soybean drought memory. We have shown that P5CS1 gene is responsible for regulating drought memory stress-induced proline accumulation. Clarifying the mechanisms of drought stress memory as well as better understand the resistance priming in plant is important to develop and maintaining crop productivity in climate change condition.

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## Conflict of interest

The authors declare no conflict of interest.

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