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DROUGHT STRESS - RELATED FUNCTIONAL CHARACTERIZATION OF TRANSCRIPTION FACTOR GmNAC085 IN SOYBEAN

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SUMMARY

Studies on soybean GmNAC085 transcription factor revealed that the gene expression in plants was induced by water shortage treatments and its overexpression in the model plant *Arabidopsis* displayed improved plant tolerance characteristics towards drought stress. In this study, we continued analyzing the biological functions of GmNAC085 using transgenic soybean system overexpressing *GmNAC085* gene, by targeting at a number of plant physiological features and biochemical activities in response to limited water growing condition. Compared to the wild-type, the transgenic line demonstrated that it possessed stress tolerance characters, including enhanced elongation of taproot, minimized reduction of shoot growth, lower intracellular H_2O_2 content and stronger peroxidase enzyme activity under drought condition. The results of this study therefore suggest the transgenic plants had better drought tolerance and the GmNAC085 plays important role in aiding plants to cope with water deficit condition, probably via regulating the growth of roots and shoots, and activities of reactive-oxygen-species- scavenging enzymes.

Keywords: *drought tolerance, GmNAC085, soybean, transgenic plant*

INTRODUCTION

Soybean (*Glycine max*) is an important crop worldwide, especially in the agricultural development in the East Asian and Pacific countries such as China, Japan, Thailand and Vietnam (Lee *et al.*, 2011). It provides high contents of protein, isoflavons and vegetable oil (Sirtori, 2001; Singh, 2010). However, the soybean growth, productivity and seed quality are heavily affected by drought stress (Manavalan *et al.,* 2009; Thao and Tran, 2012). Under such condition, various reactive oxygen species (ROS), including superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are generally built up in living cells. This accumulation led to oxidative stress which is considered as secondary stress factor following the drought stress. As a consequence, plants have to suffer cellular injuries such as lipid peroxidation, protein oxidation and nucleic acid damage (Sharma *et al.*, 2012; Jena, 2012). In addition, the disruption of cellular homeostasis by high ROS levels might also lead to the impairment of cellular activities such as photosynthesis inhibition and even cell death (Sharma, Dubey, 2005; Ciarmiello *et al.*, 2011).

Plants do naturally react against the exposed stress factor(s) to protect themselves as much as they can, by deploying a series of responsive/adaptive mechanisms related to the change in anatomy, physiology, biochemistry and genetic regulation (Shao *et al.,* 2007). Among these, using enzymes to scavenge the excessive ROS out of plant cells is a common defending strategy. For example, peroxidase (POD) and catalase (CAT) are responsible for removing H_2O_2. Therefore, analyzing activities of these enzymes is one of important parameters for evaluating the stress degree and stress tolerance capacity in plants of interest.

To deal with the sensitivity of soybean to water limitation condition, enhancement of drought stress tolerance in soybean by genetic engineering has been considered a solution. In recent decades, technological developments and intensive research in model and crop plants have revealed the involvement of many transcription factors in regulating plant adaptation to drought stress, among which are many

members of NAC (NAM, ATAF, CUC) family (Nuruzzaman *et al.*, 2010; Le *et al.*, 2011; Hussain, 2017). In general, NACs are known as plant-specific transcriptional factors that regulate various plant developmental processes such as shoot apical meristem formation and maintenance (Weir *et al.,* 2004); floral development and morphogenesis (Sablowski and Meyerowitz, 1998); embryo development (Duval *et al.,* 2002); hormone signaling (Xie *et al.,* 2000; Fujita *et al.,* 2004) and regulation of secondary cell wall synthesis (Ko *et al.,* 2007).

Following the identification of involved NACs in plant response to water deficit stress conditions, a significant number of studies reported the improved drought or dehydration tolerance when manipulating the expression of different *NAC* genes obtained from different species, mainly by overexpressing the target gene. Several typical examples are *Arabidopsis NAC* genes *ANAC019*, *ANAC055*, *ANAC072* (Tran *et al*., 2009), and *ATAF1* (Liu *et al*., 2016); rice *NAC* genes *SNAC1* (Liu *et al*., 2014), *SNAC3* (Fang *et al*., 2015), *OsNAC5* (Song *et al*., 2011), and *ONAC022* (Hong *et al*., 2016); wheat *NAC* genes *TaNAC2a* (Tang *et al*., 2012), *TaNAC67* (Mao *et al*., 2014) and *TaNAC69* (Xue *et al*., 2011); maize *NAC* gene *ZmSNAC1* (Lu *et al*., 2012); and soybean *NAC* gene *GmNAC085* (Nguyen *et al*., 2018).

In our study, we focused on evaluating the effects of *GmNAC085* overexpression on several physiological and biochemical traits in soybean plants when they were grown under normal and drought conditions. Expression of *GmNAC085* was shown to be induced by dehydration stress in Williams 82 soybean cultivar (Le *et al.*, 2011), DT51 and MTD720 soybean cultivars (Hieu *et al*., 2016). Meanwhile, another report revealed that the gene activity was also up-regulated in drought-treated soybean plants (Thao *et al*., 2013; Thu *et al.,* 2014). According to their results, *GmNAC085* was suggested to play important role of in supporting plant response to drought stress since its expression was found to be increased at a much higher level in the drought-tolerant soybean cultivar when compared to its corresponding level in a drought-sensitive cultivar. Most recently, further investigation on function of GmNAC085 using transgenic model plants showed that the *Arabidopsis* overexpressing *GmNAC085* displayed improved drought tolerance, probably due to stronger antioxidant capacity (Nguyen *et al*., 2018). Therefore, the results obtained from our research using transgenic crop plant system would provide a clearer picture about the role of GmNAC085 in regulating plant response to water deficit condition as well as its potential application in plant genetic engineering.

MATERIALS AND METHODS

Plant materials and growing condition

The wild-type seeds W82 (WT) were received from Vietnam Legumes Research and Development Center (Vietnam) and transgenic soybean seeds (Williams 82 seeds harboring 35S:*GmNAC085* and selectable marker *bar* gene) (Trans) were generated by using the *Agrobacterium*-mediated transformation method taken from the University of Missouri (USA). The plants at V4 stage (22 days after germination) were sprayed with BASTA (glufosinate ammonium) (Wako, Japan) (80 mg/L, 3-ml dose per plant). After 3 days, the transgenic plants should remain healthy and green while the non-transgenic plants would display yellow, paled and/or wilted leaves. Following Mendelian laws, the transgenic line carrying one copy of transgene in the homozygous form was identified after screening 4 consecutive generations (Hai *et al.*, 2017). All plants were grown under net house condition (30°C day-time/28°C night-time, 12h light/12h dark photoperiod, and humidity 60–70%).

Shoot growth and root growth assay

Four-day-old seedlings grown in elongated plastic tube (80 cm in height and 10 cm in diameter) filled with Tribat soil (Saigon Xanh Bio-Technology Ltd. Company, Vietnam), which had similar size, were selected for drought-induced treatment experiment. Regular irrigation was discontinued after 12 days of planting to initiate the 15-day-drought stress treatment. The soil moisture contents (SMC) were monitored at 5-day intervals using moisture meter (Total Meter, Taiwan). For control, another set of plants was maintained under well-watered conditions. After 27 days of planting, the whole root systems from both drought-stressed and well-watered groups were gently removed from soil. Each plant was used for measuring the lengths of taproot and main shoot. Then the plant materials were dried at 65°C for 48 h to obtain the dry biomass weights of shoot and root tissues.

Determination of cellular H_2O_2 level

The H_2O_2 content was determined according to method described in Patterson *et al.* (1984). In brief, a 21-day-drought treatment was applied to 14-day-old plants. Then, the leaf sample tissues were collected at specific time-points for analyzing cellular H_2O_2 content. For H_2O_2 extraction, 0.2 g of leaves were ground in 2 mL phosphate- buffered saline (PBS; 0.1 M, pH 7.4) on cold mortar and pestle. The crude extract was centrifuged at 10,000 rpm for 10 minutes at 4°C. Next, 1 mL of extraction of cellular H_2O_2 was mixed vigilantly with 0.1% Titanium Sulfate in 20% H_2SO_4 (v/v). The absorbance of supernatant was measured at 410 nm by spectrophotometer after centrifuging at 12,000 rpm for 10 minutes at room temperature for complete reaction. The mixture of 1 mL of PBS with 1 mL of 0.1% Titanium Sulfate in 20% H_2SO_4 (v/v) was used as blank. Three biological replications were used for each line. A standard curve for H_2O_2 was prepared to infer the cellular hydrogen peroxide content.

Peroxidase (POD) activity measurement

To determine the enzymatic activity, a crude enzyme extract was prepared by homogenizing 0.2 g of leaf tissue in 2 mL cold extraction buffer (pH 7.0) containing 1 mM EDTA and 2% Polyvinyl-pyrrolidone (PVP-8000) in 50 mM Potassium phosphate buffer using a pre-chilled mortar and pestle. After that, the homogenate was centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant was then used for determination of total protein content and POD activities.

The total protein was quantified by Bradford (1976) method. The POD activity was determined according to Shannon *et al.* (1966) and calculated by the formula of Rodríguez *et al.* (2001). Shortly, the reaction mixture (pH 5.4, temperature of 37°C) consisted of 3 mL of 0.1 M acetate, 0.04 mL of 0.1 M H_2O_2, 0.04 mL of O-dianisidine 0.2% (Acros, USA) and 100 µL of plant extract. Blank was prepared with all components in reaction mixture except the replacing the enzyme extract by the enzyme extraction buffer. The absorbance of mixture was recorded instantly for initial optical density value and after three minutes for final one. There were three biological replications for each line to be studied (n=3).

Statistical analyses

The data were analyzed by Student's t-test to identify the statistical significance with p-value < 0.05.

RESULTS AND DICUSSION

Root and shoot characters under normal growing condition

When performing the shoot and root assay, the soil moisture content (SMC) was monitored to ensure the appropriate set-up for our experiment. According to the SMC record, the soil moisture was maintained well around 80% for plants that were grown under normal condition with adequate irrigation (Fig. 1e). Under non-stress growing condition, the transgenic soybean line and the WT counterparts had no difference in shoot growth or shoot biomass accumulation (Fig. 1a, c). Nevertheless, an interesting feature found from this study was that the overexpression of *GmNAC085* caused a much shorter tap-root in the transgenic than in the WT when the plants were grown under normal condition. According to our results, the average lengths of tap-roots in the transgenic and WT were 58.7 cm and 67.2 cm, respectively (p-value < 0.05) (Fig. 1b). Previously, research on *GmNAC085*-overexpressing *Arabidopsis* also reported the growth retardation recognition in both root and shoot tissues of the transgenic (Nguyen *et al*., 2018). However, the data obtained from our study revealed similar average root biomass in both these genotypes. This could be explained by the compensation of more lateral roots in the transgenic soybean line (Fig. 1d).

Changes in Shoot and Root-related traits upon drought stress

When assessing the drought tolerance capacity in plants, root and shoot growth rates are considered important traits that should be examined (Huang *et al.*, 2009; Thao *et al*., 2013). According to our analyses, similar to results from previous reports, the drought stress applied to vegetative growth stage of plants resulted in the inhibition of shoot growth yet the induction of root growth in both genotypes (Fig. 1). Generally, it has been agreed that plants grown in soil with low water availability tend to reduce should growth to retain the water potentials and prioritize plant survival (Sharp *et al.*, 2004), expand its root system to maintain water supply for plants' needs (Sponchiado *et al.*, 1989).

Look at the data in more details, regarding the shoot trait, the negative effects of drought on the transgenic soybean line was not so serious as those

in the WT plants since the significant reduction in shoot growth and shoot biomass were only seen in the latter (Fig. 1a, c). Meanwhile, there was no difference between the average taproot lengths of the two tested genotypes under drought stress, even though the transgenic had much lower mean of taproot length in adequately watering condition. As seen from Fig. 1b, 15-day drought treatment induced the root elongation at a higher rate in the transgenic (21%) than the rate in WT plants (8%) when compared to the root growth rate of the same genotype grown under normal condition.

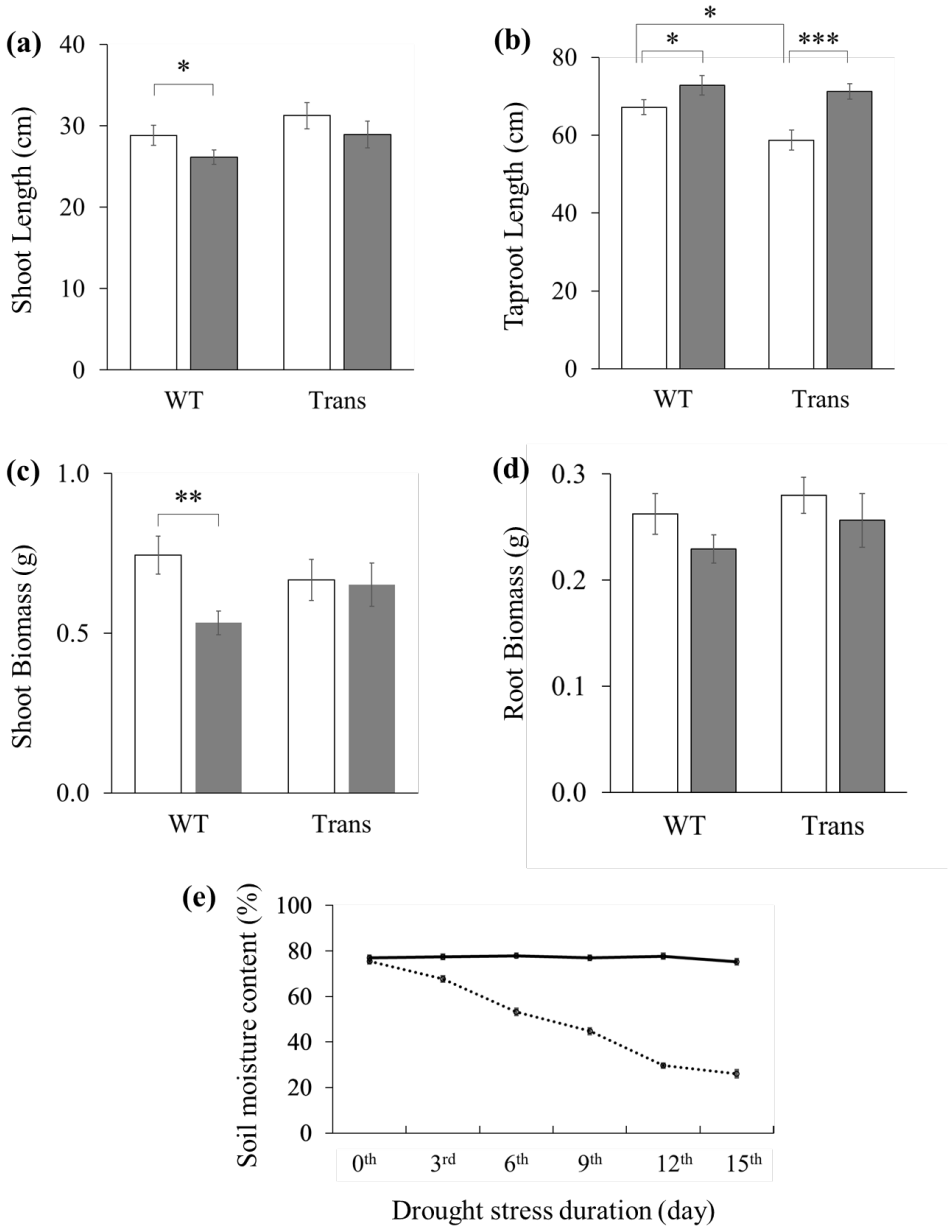

Figure 1. The shoot and root development under normal (white bars) and drought (grey bars) conditions of *GmNAC085*-overexpressing transgenic (Trans) and the wild-type (WT) soybean plants (n=9/). For drought treatment, water withholding was applied to 12-day-old plants for 15 days. (a) Shoot length; (b) Taproot length; (c) Shoot biomass; (d) Root biomass; (e) Soil moisture content was measured under well-watered (bold line) and drought (dash line) conditions (n=3). Error bars represent standard errors, Student's *t*-test was used to evaluate if the difference was significant (* *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value <0.001).

Evaluation of plant stress degree based on biochemical parameters

In addition to the shoot- and root- related traits, we also analyzed few common biochemical parameters in order to evaluate the potential drought tolerance capacity of the transgenic plant more precisely. Firstly, the accumulation of non-radical species H_2O_2 in leaf tissue upon the drought treatment was assessed. Hydrogen peroxide in plants functions as a double-edged sword whereby it confers certain advantages at low concentrations but become harmful at excessive accumulations. For example, at low intracellular concentrations, H_2O_2 plays as a regulatory signal for critical physiological processes such as photorespiration and photosynthesis, stomata movement, cell cycle and growth and development (Das and Roychoudhury, 2014). Under osmotic stress conditions, high hydrogen peroxide can cause deleterious effects to cell structure and activities (Dat *et al.*, 2003). According to the obtained results in this study, 21-day water withholding induced considerably the level of H_2O_2 in plant cells, upon which 2.3-fold and 2-fold increase was seen in this kind of ROS in the WT and *GmNAC085*-overexpressing transgenic plants, respectively (Fig. 2b, *p*-values <0.001). A significant lower accumulation of H_2O_2 by 26% (*p*-value <0.05) in the transgenic plants than the WT indicates that either the former was less stressed or possessed a more efficient system to remove the hydrogen peroxide out of the cells. This also suggests for a possible weaker damage effects by stress on the transgenic soybean line.

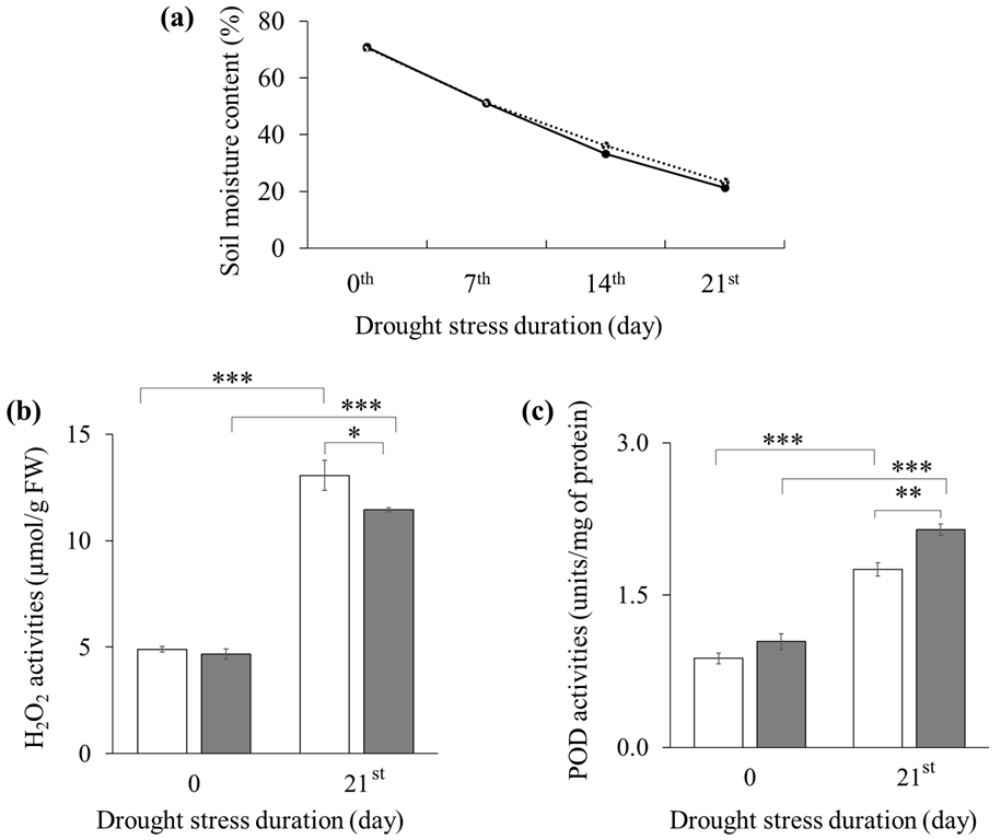

Figure 2. H_2O_2 content and peroxidase enzyme activities in *GmNAC085*-overexpressing transgenic (grey bars) and the wild-type (white bars) plants upon drought stress exposure. The drought condition was applied for 14-day-old plants. (a) Monitored soil moisture content over the course of course of drought treatment (bold line for WT and dash line for transgenic, n=3); (b) cellular H_2O_2 content; (c) peroxidase (POD) activity. Error bars represent standard errors, Student's *t*-test was used to evaluate if the difference was significant (* *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value <0.001).

We next moved to measure the peroxidase activity, one kind of enzyme that is responsible for scavenging the H_2O_2 and known as one of the first enzymes increasing in activities in plants upon stress stimulation (Vicuna, 2005). In both genotypes used in this study, the drought stress triggered a substantial increase in POD activity (*p*-values < 0.001) (Fig. 2c). Although no clear advantage in POD activity seen in the transgenic line compared to that of the WT counterpart growing at normal condition, performance of this enzyme was significantly better (approximately 20% higher in POD activity) under water deficit condition, implying that the lower H_2O_2 seen in the transgenic probably due to effective action of POD.

Taking the physiological and biochemical results together, it is suggested that the transgenic plants overexpressing *GmNAC085* might confer better tolerance to drought stress. The biological function of this NAC transcription factor might be involved in regulating antioxidant activities and shoot-/root-related traits in plants.

CONCLUSION

In this study, we have investigated the effects of *GmNAC085* overexpression to the change in plant shoot and root growths, cellular hydrogen peroxide content and peroxidase activity under drought growing condition. According to the obtained results, the transgenic soybean line displayed better drought tolerance potential, thus indicating adaptive regulatory function of *GmNAC085* under drought stress such as promoting root development and activity of H_2O_2-scavenging peroxidase. These preliminary findings encourage more in-depth studies in the future to fully elucidate the functions and its acting mechanism of GmNAC085 as well as the potential to improve plant tolerance by manipulating the expression of *GmNAC085*.

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PHÂN TÍCH CHỨC NĂNG CỦA NHÂN TỐ PHIÊN MÃ GmNAC085 TRONG ĐÁP ỨNG STRESS HẠN Ở CÂY ĐẬU TƯƠNG

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TÓM TẮT

Các nghiên cứu về nhân tố phiên mã GmNAC085 ở đậu tương cho thấy cây có biểu hiện gen tăng cường khi bị xử lý thiếu nước và ở cây mô hình *Arabidopsis* biểu hiện vượt mức GmNAC085 thì có các đặc điểm chống chịu hạn tốt hơn. Ở nghiên cứu này, chúng tôi tiếp tục tìm hiểu chức năng của GmNAC085 thông qua sử dụng hệ thống cây đậu tương chuyển gen có biểu hiện vượt mức *GmNAC085*, tập trung vào phân tích một số chỉ số sinh lý và sinh hóa ở cây khi được trồng dưới điều kiện thiếu nước. Kết quả thu được cho thấy, so với cây không chuyển gen thì cây chuyển gen có các tính trạng chống chịu stress như tăng cường dài rễ, giảm thiểu ảnh hưởng tới chiều cao và sinh khối của thân, có hàm lượng hydrogen peroxide nội bào thấp hơn và có hoạt tính enzyme peroxidase cao hơn khi bị trồng ở điều kiện thiếu nước. Những phân tích này cho thấy cây chuyển gen có tiềm năng chịu hạn tốt hơn và GmNAC085 có thể đóng một vai trò quan trọng giúp cây đối phó với tác động của hạn hạn, khả năng là thông qua tác động lên sự tăng trưởng của mô chồi và rễ cũng như hoạt động của enzyme khử các gốc oxy hóa tự do.

Từ khóa: *chống chịu hạn, GmNAC085, đậu tương, cây chuyển gen*