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EXPRESSION ALTERATION ANALYSES IN THE TRANSGENIC *ARABIDOPSIS* CARRYING SOYBEAN *HISTIDINE-CONTAINING PHOSPHOTRANSMITTER* GENE UNDER SALINITY STRESS CONDITION

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SUMMARY

Productivity of many crops is highly vulnerable to extreme external conditions. Environmental stress factors such as drought and salinity have become more and more serious due to climate change and appear in many areas worldwide with higher frequency. As both drought and salinity belong to osmotic stress, they have similar negative effects on plant growth, development, and productivity as well as trigger similar stress responses by plants. In a previous study analyzing the expression profile in two soybean (*Glycine max*) cultivars with contrasting drought-tolerant phenotypes, a member of two-component system (TCS) in soybean, *GmHP08*, was proposed to associate with the plant tolerance capacity to drought. Subsequent *in planta* study confirmed its action as a positive regulator under drought conditions, as the transgenic *Arabidopsis* plants ectopically expressing *GmHP08* acquired better drought tolerance. Following this, the presented research further explored the possible function of GmHP08 in mediating plant response to salinity. The obtained data from RT-qPCR analyses suggested that GmHP08 might positively enhance the salt tolerance of the *Arabidopsis* transgenic plants by altering the transcriptional abundance of several stress-related genes, including *RD29A*, *RD29B*, *ABI5*, *SAG13*, and *CSD1*. Activities of these genes are known to be associated with osmoprotection, senescence process, and antioxidation, which contribute to salt-tolerance ability of the transgenic plants. These results provided the first line of molecular evidence regarding GmHP08 function in plant response to salinity conditions. Therefore, extensive studies should be conducted in future studies to elaborate on the mechanisms by which this TCS member could improve various types of osmotic stress tolerance in plants.

Keywords: *Arabidopsis*, *GmHP08*, RT-qPCR, salt tolerance, two-component systems

INTRODUCTION

Salinity is considered as one of the major abiotic stress factors that not only reduces plant growth and productivity but also accelerates the cutting-down of land usage (Gong *et al*., 2020). According to a recent report, soil salinization affects almost one-fifth of the cultivated land all around the world, especially in the flooded or saltwater-intrusive areas (Morton *et al*., 2019). Negative impacts of salinity on plant growth and development mainly come from three types, which are (i) water deficit stress (i.e. dehydration) due to reduced water potential in soil causing difficulty of water uptake by plant root system, (ii) ion toxicity due to excessive accumulation of specific ions such as Na^+ and Cl^- in plant cells, and (iii) oxidative stress due to overproduction of endogenous reactive oxygen species (ROS) under the adverse condition (Munns, 1993; Chaitanya *et*

al., 2003). Under prolonged salinity conditions, plants suffer decreased photosynthetic efficiency and yield loss, due to disturbed activities of carbon-reduction cycle and light reactions, growth retardation as well as promoted senescence process (Lawlor, Tezara, 2009).

In recent decades, many studies have been conducted to investigate the mechanisms and the pathways that plants utilize to respond to abiotic stresses including salinity (Gong *et al*., 2020). So far, various resistant mechanisms have been identified in plants, which involve anatomical, physiological, biochemical and molecular adjustments, with the engagement of diverse signaling transduction pathways (Cramer *et al*., 2011). Among these include the two-components system (TCS), which exists not only in plants but also in other group species such as bacteria and fungi. The action of the TCSs confers the plants capabilities to sense and respond to environmental stimuli (Thu *et al*., 2015). The simplest form of a TCS contains two basic components, which are a sensor histidine kinase (HK) that receives the input signal, and an effector response regulator (RR) that delivers the signal to regulate the expression of its downstream target genes (Hwang *et al*., 2002). There are also other complex forms of the TCS that have an extra component, known as histidine-containing phosphotransfer (HPt). This is an intermediate protein connecting the phosphor transfer from the HKs to the RRs, which is referred to as multistep histidine-aspartate phosphorelay (Schaller, 2000; Lohrmann, Harter, 2002). Additionally, several TCS members were identified to participate in abiotic stress response. For example, *Arabidopsis* HK1 (AHK1), *Arabidopsis* HP2 (AHP2), AHP3 and AHP4 were shown to act as positive and negative regulators under drought stress conditions, respectively (Tran *et al*., 2007; Wohlbach *et al*., 2008; Tran *et al*., 2010).

Due to its economic and nutritional importance, soybean (*Glycine max* L. Merrill) is one of the most essential crops worldwide (Andres *et al*., 2009; Le *et al*., 2012). It is, however, very susceptible to drought and salinity, thus suffers a significant decrease in productivity (Wang *et al*., 2016). Previously, nine soybean TCS-related genes including *GmHK07*, *GmHK16*, *GmHP08*, *GmRR04*, *GmRR16*, *GmRR32*, *GmRR34*, *GmPRR39*, and *GmPRR44* which might potentially contribute to the drought tolerance capacity in plants have been identified (Le *et al*., 2011; Thu *et al*., 2015). Regarding *GmHP08*, it was found that expression of this gene was significantly induced in the soybean shoot tissue after 10 hours of dehydration treatment (Le *et al*., 2011) or under 15-day-drought stress conditions (Thu *et al*., 2015). Subsequent *in planta* study indicated that transgenic *Arabidopsis* carrying *GmHP08* acquired better drought tolerance (Chuong *et al*., 2021), confirming the critical role of this protein in response to drought.

As drought and salinity cause similar impacts on plant growth and development, which particularly results in osmotic stress and oxidative stress (Munns, 2002; Uddin *et al*., 2016), the role of *GmHP08* in plant response to salinity is of interest for investigation. By utilizing transgenic *Arabidopsis* carrying *GmHP08*, the assessment of salt-tolerance related to GmHP08 was carried out in this study, based on expression analyses of several key osmotic stress-related genes. These were two well-known marker genes [*Responsive to desiccation 29A* (*RD29A*), *RD29B*], one regulatory gene [*ABA-insensitive 5* (*ABI5*)], one senescence-related gene [*Senescence-associated gene 13* (*SAG13*)], and one antioxidant enzyme-encoding gene [*Superoxide dismutase [Cu-Zn] 1* (*CSD1*)]. According to previous studies, expression of *RD29A* and *RD29B*, which belong to *Late embryo abundance* (*LEA*) family, was enhanced by drought, cold, abscisic acid (ABA) and high salinity conditions (Jin *et al*., 2013; Li *et al*., 2013; Zhou *et al*., 2015). The third selected gene for examination, *ABI5*, is a basic leucine zipper-typed transcription factor. It functions in the core of the ABA signaling, known as to play a crucial role in controlling seed germination, post-germination growth (Skubacz *et al*., 2016) and also participate in regulating plant responses

to adverse environmental conditions such as drought and salinity (Finkelstein, Lynch, 2000; Nakamura *et al*., 2001). Meanwhile, relative expression of *SAG13* normally increases when plant aging is accelerated such as under stress conditions (Huang *et al*., 2015). The last chosen gene in our study, *CSD1*, encodes superoxide dismutase (SOD) [Cu-Zn], which is an important antioxidant enzyme acting in eradication of excessive superoxide (a type of ROS) from plant cells (Jagadeeswaran *et al*., 2009).

MATERIALS and METHODS

Materials

The wild-type (WT) *Arabidopsis thaliana* ecotype Col-0 was utilized in this research as control and as material to generate transgenic plants carrying *GmHP08* under the regulation of *Cauliflower mosaic virus (CaMV) 35S* promoter. The procedures for construction of recombinant vector carrying *35S::GmHP08*, plant transformation as well as selection of homogenous transgenic plant progenies were described in our previously published study (Chuong *et al*., 2021).

Plant growth

The seeds of transgenic and WT plants were sterilized using 70% ethanol and 10% Javel before being sown on germination medium (GM) (Murashige and Skoog medium supplemented with 1% glucose and 0.8% agar, pH 5.8). They were then incubated for 2 days in dark and cold (4 ºC) environment for breaking seed dormancy. After that they were cultivated in normal growth conditions (22 ºC, 16-h-day/8-h-night period).

Salt-stress assay

The WT and transgenic plants were treated with NaCl following methods in previous studies (Li *et al*., 2014; Jiang *et al*., 2015) with some modifications. In brief, fourteen-day-old WT and transgenic plants were transferred from the GM to water-saturated soil and grown under normal conditions for the next 16 days. After that, the salt-stress assay was applied by irrigating 120 mL of 200 mM NaCl to each tray every 2 days. The aerial parts of plants were harvested at day 0, day 3rd and day 7th since salt application by freezing in liquid nitrogen. For each time point of sample collection, three biological replicates were used for each genotype.

Total RNA extraction, cDNA synthesis and reverse-transcription quantitative PCR

Pure total RNA of all collected samples were obtained by using commercial kits (Thermo Fisher Scientific, USA) for RNA extraction and DNA removal (Thao *et al*., 2013). After this, cDNA synthesis was performed using 1,000 ng of RNA from each sample and following the instruction of the kit manufacturer (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, USA).

RT-qPCR reactions were prepared in 25 µL of total volume, which contained SYBR Green PCR Master mix (Thermo Scientific), primers (0.4 µM each) and 1 µL cDNA. The PCR thermal profile was established in accordance with our previous study (Thao *et al*., 2013).

Actin 2 (*ACT2*) (Yang *et al*., 2016) was used as the reference gene for gene expression analysis. The sequences of primers for five target genes were obtained from previous studies including *RD29A* (Rasheed *et al*., 2016), *RD29B* (Liu *et al*., 2018), *ABI5* (Huang *et al*., 2015); *CSD1* (Chen *et al*., 2013) and *SAG13* (Huang *et al*., 2015). The relative transcript abundance target genes between WT and transgenic plants under different conditions was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak, Schmittgen, 2001).

Statistical data analysis

The obtained results were analyzed by Student's *t*-test. The significant difference was confirmed if the *p*-value was below 0.05 (Thu *et al*., 2015).

RESULTS AND DISCUSSION

Expression of *RD29A* and *RD29B*

Upon being irrigated by salt-water, a significant

increase in transcript abundance of *RD29A* and *RD29B* was observed in both transgenic and WT plants (Figure 1), especially after 7 days. Particularly, *GmHP08*-transgenic plants displayed a much higher induction level in expression of these genes, compared with that in the WT plants at the same time point of analysis under the stressed conditions. To be specific, the expression levels of *RD29A* were 3.39-fold higher after 3 days and 1.95-fold higher after 7 days in the transgenic plants. Meanwhile under normal conditions, although expression levels of *RD29A* were comparable between the two genotypes (Figure 1A), that of *RD29B* was higher in the WT plants than in the transgenic plants by 1.47-fold (Figure 1B).

According to literature, *RD29* genes have been highlighted as markers for plant response to dehydration and high salinity conditions, with an increase in gene expression (Yamaguchi-Shinozaki, Shinozaki, 1994; Msanne *et al*., 2011). Although the function of hydrophilic proteins encoded by these genes remained elusive, they share similarity to LEA proteins. Therefore they are called LEA-like proteins and suggested to have similar function with LEA proteins (Yamaguchi-Shinozaki, Shinozaki, 1993a; Yamaguchi-Shinozaki, Shinozaki 1993b; Msanne *et al*., 2011). LEA proteins are responsible for osmoprotection, along with other elements such as osmotin, chaperones, sugars and proline (Shinozaki, Yamaguchi-Shinozaki, 2007; Msanne *et al*., 2011). Therefore, the higher expression of *RD29A* and *RD29B* in *GmHP08*-transgenic plants might confer them better osmoprotection under salinity conditions (Figure 1).

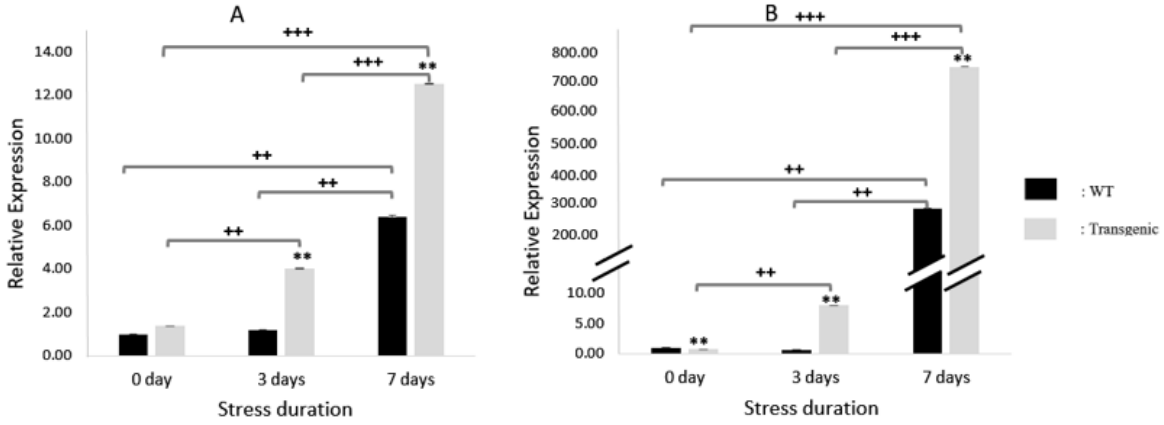

Figure 1. Relative expression of marker genes *RD29A* (A) and *RD29B* (B) in wild-type (WT) and transgenic plants under normal (0 day) and salinity conditions. Significant difference in expression between conditions for the same genotype was shown by the plus symbol above the drawing lines (**++**: *p*-value < 0.01, **+++**: *p*-value < 0.001) and between the two genotypes under the same condition by the star symbol displayed above the transgenic bar (**: *p*-value < 0.01).

Expression of *ABI5, SAG13* and *CSD1*

When exposed to the salt stress condition, *ABI5* expression significantly increased in both transgenic and WT plants (Figure 2A), especially after 7 days with higher level in the transgenic than in the WT plants. Meanwhile, *SAG13* expression levels were only induced in both studied genotypes after 3 days of the stress application. At the stage of one-week treatment, further enhancement in *SAG13* expression was only observed in the *GmHP08*-transgenic plants but not the in WT plants, thus leading to substantially higher expression level of *SAG13* in the former group over its WT counterparts at this time point **(**Figure 2B**)**. With the expression pattern of *CSD1*, this gene was always expressed more highly in the ectopic expression line than in

the WT plants at the same time point of analysis, in both growing conditions. It is noticed that compared to the non-stressed WT plants, the expression of this gene after 3-day stress treatment only slightly increased and followed by a significant decrease after 7 days of treatment. Meanwhile, the expression levels of *CSD1* in the transgenic plants increased significantly after 3-day exposure to salinity before dropping to almost similar level at day 0 (Figure 2C).

Among *ABI5*, *SAG13* and *CSD1*, under non-stressed condition, transcriptional levels of *ABI5* between the two genotypes were similar (Figure 2A), whereas those of the other two genes showed a significant difference between the transgenic and WT plants (Figures 2B, 2C). Under salinity conditions, expression of *ABI5* was 1.6-fold higher after 3 days and 1.9-fold higher after 7 days than the corresponding levels in the WT counterparts (Figure 2A). In contrast, the expression of *SAG13* was higher in the WT plants than in transgenic plants by 2.9-fold after 3 days, although after 7 days, the reverse trend was observed (i.e. *SAG13* expression was significantly more upregulated in the transgenic plants than in WT plants by 1.54-fold) **(**Figure 2B). For *CSD1*, its expression showed an increase in transcript abundance, which was higher in the transgenic plants in both time points of analyses (3rd and 7th day) under salinity, with the higher levels approximately about 1.6 and 2.4-fold, respectively (Figure 2C).

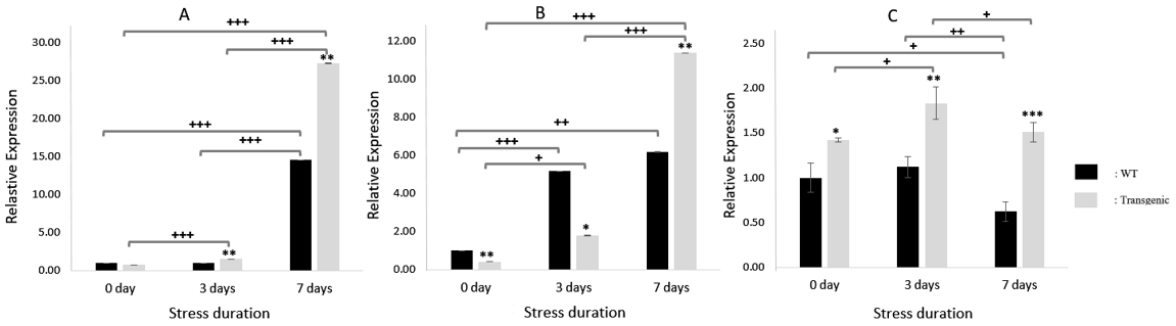

Figure 2. Relative expression of genes *ABI5* (A), *SAG13* (B) and *CSD1* (C) in wild-type (WT) and transgenic plants under normal (0 day) and salinity conditions. Significant difference in expression between conditions for the same genotype was shown by the plus symbol above the drawing lines (**+**: *p*-value < 0.05, **++**: *p*-value < 0.01, **+++**: *p*-value < 0.001) and between the two genotypes under the same condition by the star symbol displayed above the transgenic bar (*: *p*-value < 0.05, **: *p*-value < 0.01, ***: *p*-value < 0.001).

ABI5 is normally known as an inhibitory regulator of plants during seed germination but positive regulator in processes of protection, water retention and toxicity isolation in the chloroplasts as well as in preventing cellular damage when being exposed to adverse conditions (Lopez-Molina *et al*., 2001; Brocard *et al*., 2002; Skubacz *et al*., 2016). Thus, an increase in transcriptional level of this gene might assist the *GmHP08*-transgenic plants to have a better cellular protection and higher possibility to survive under prolonged high salinity conditions in comparison with the WT plants. With SAG13, this protein acts as a marker of senescence in *Arabidopsis* and other plant species (Brodersen *et al*., 2002; Espinoza *et al*., 2007). It was also reported that SAG13 plays a role in germination process, seedling development under oxidative stress and has a crucial role in mediating plant response to ROS attack in combination with light stress. Furthermore, the presence of SAG13 might assist accumulation of anthocyanin – an important compound not only in reproduction but also in protection during oxidative stress (Liu *et al*., 2018; Dhar *et al*., 2020). Therefore, based on the

obtained data, it is hypothesized that *GmHP08*-transgenic plants might have delayed senescence under short stress duration conditions for maintaining photosynthesis but promoted leaf senescence under prolonged stress conditions to prioritize their survival (Figure 2B).

Under stress conditions, plants rely on antioxidant molecules and enzymes to scavenge the excessive ROS contents in plant cells, as accumulation of these species can result in the disruption of cellular structure and activities (Foyer *et al*., 1994; Mittler, 2002). Regarding the ROS-type superoxide, its detoxification is achieved by the activities of SOD enzymes (Mittler, 2002). Many studies have shown that the transgenic plants acquired better tolerance to salinity with increased expression of SOD-related genes and SOD enzyme activities (Hu *et al*., 2012; Chen *et al*., 2017). Thus, the increase in *CSD1*, which is an SOD-encoding gene, in the *GmHP08*-transgenic plants suggests enhanced SOD activity and better superoxide removal in these plants, thus conferring a better protection from salinity-induced oxidative stress.

CONCLUSION

Taken together, the obtained results from this study demonstrate that the transgenic plants ectopically expressing *GmHP08* have higher salt-tolerance capacity by upregulating the transcriptional level of several important stress-responsive genes. Hereby, GmHP08 was found to act as a positive regulator of *RD29A*, *RD29B*, *ABI5*, *SAG13* and *CSD1*. This conferred the transgenic plants certain advantages in osmoprotection and endogenous ROS removal, and thus potentially better tolerance to salinity stress. Nevertheless, comprehensive understanding of GmHP08 function is required to serve for the thorough evaluation of its application potential in stress tolerance improvement. The future research should be focused on how the transgenic plants react with different concentrations of salt as well as on expression of other stress-responsive genes in connection with physiological and biochemical data analyses.

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