EXPRESSION STUDY OF STRESS-RELATED GENES IN SALINITY-TREATED TRANSGENIC ARABIDOPSIS HARBORING SOYBEAN RESPONSE REGULATOR 34

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SUMMARY

Owing to their sessile nature, plants are easily affected by various external factors. Among these, drought and salinity are considered as the most common stresses, which often pose a threat to plant growth and development. Major effects of the drought and salinity are interconnected and drive similar series of molecular changes in plants. These alterations in response to the stress are under the regulation of various signaling pathways, including the engagement of evolutionarily conserved two-component systems (TCSs). Three components with distinct functions can be found in a functional TCS, which are histidine kinases (HKs), histidine-containing phosphotransfer proteins (HPTs), and response regulator proteins (RRs). Previous research revealed that the soybean (Glycine max) GmRR34 acts as an important regulatory protein in plants under drought stress conditions. In this project, the investigation on the role of GmRR34 in osmotic stress responses was extended to salinity by examining the expression of a subset of salinity-responsive genes using RT-qPCR method. Our analyses showed that the transgenic Arabidopsis plants ectopically expressing GmRR34 displayed enhanced expression of several important stress-related genes, including Catalase 1 (CAT1), Stomatal ascorbate peroxidase 1 (sAPX1), Copper/zinc superoxide dismutase 1 (CSD1), Sodium/hydrogen exchanger 1 (NHX1) and Salt overly sensitive 2 (SOS2). These results indicate that GmRR34-transgenic plants might be more salt-tolerant thanks to stronger activities of antioxidant enzymes and better capacity in maintaining cytosolic ion homeostasis. Therefore, it is highlighted the necessity to perform further studies to fully characterize the GmRR34 biological functions as well as explore its application potential in enhancing the salt tolerance of crop plants.

Keywords: Arabidopsis thaliana, gene expression, GmRR34, salt stress, two component system

INTRODUCTION

Being sessile organisms, plants have to confront a wide range of adverse environmental factors. Among the abiotic stress conditions, soil salinization has become more and more serious and is predicted to affect approximately 6% of the global land area and 20% of the irrigated land (Ghonaim et al., 2021). The high salt levels in soil coming from accumulation of certain ions such as Na⁺ and Cl⁻ can change the water status, ionic concentration as well as reactive oxygen species (ROS) contents in the plant tissues, which lead to the disruption of various biological activities of the plants (Shrivastava, Kumar, 2015). Therefore, plant growth, development as well as productivity in terms of quality and quantity are negatively affected (Ghassemi-
Golezani, Taifeh-Noori, 2011). As soil salinization tends to be intensified by climate change and water intrusion from the sea into the mainland, comprehensive understanding of mechanisms by which plants utilize to adapt to salinity conditions is essential for the production of elite cultivars with better salt tolerance, thus ensuring the food security and stable economy.

Over the course of evolution, plants have diversified mechanisms to perceive and transduce external stimuli, which allow them to counteract the negative effects of the stressors (Rejeb et al., 2014). Over the past two decades, significant progress has been made in understanding the stress-signaling pathways in plants (Yang, Guo, 2018). Among those, two-component systems (TCSs) are considered as one of the major routes that can regulate various hormonal and environmental signaling events (Grefen, Harter, 2004). A basic TCS comprises of two main proteins - a sensor histidine kinase (HK) and a response regulator (RR). The HK obtains the input signal, which will lead to the phosphorylation of its conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartate residue of the RR, activating this protein to regulate expression of its downstream target genes (Thao et al., 2013). In more complicated TCSs, a third component, known as histidine-containing phosphotransmitter (HP/HPt), serves as a phospho-relay intermediate between the hybrid HK and the RR (Urao et al., 2000).

Many studies have reported on the involvement of TCSs in regulating plant stress response. For example, it is demonstrated that the Arabidopsis HK1 (AHK1) is a positive regulator of the abscisic acid (ABA)-signaling transduction pathway as well as drought and salt stress responses (Tran et al., 2007). In soybean (Glycine max), at least 21 HKs, 13 HPs and HP-like proteins, and 46 RRs/pseudo RRs (PRRs) have been identified from the plant genome (Mochida et al., 2010; Schmutz et al., 2010). Among those, there are 9 genes (2 HKs, 1 HP and 6 RRs) have been found to potentially associate with the drought tolerance capacity of plants (Thu et al., 2015). In particular, in planta functional characterization of GmRR34, one gene in this list, has shown that this soybean RR can significantly enhance plant tolerance toward the drought stress (Nghia et al., 2020). It has been known that water deficiency and salinity both cause osmotic and oxidative stresses as well as trigger similar responses in plants (Okon, 2019). Therefore, in this study, we analyzed the expression of a subset of the stress-related genes using the drought-tolerant Arabidopsis ectopically expressing GmRR34 under salinity stress conditions. The findings would provide preliminary data to evaluate if GmRR34 functions as a positive regulator mediating plant response to multiple abiotic stresses such as drought and salinity.

MATERIALS AND METHODS

Plant materials

The cDNA of soybean GmRR34 was cloned into vector pBI121 and placed under the control of promoter CaMV 35S (Nghia et al., 2020). The recombinant vector was transferred into Agrobacterium, followed by transformation into Arabidopsis (Col-0) as described in previous study (Zhang et al., 2006). The transgenic line used in this study has been verified to carry homozygous, single copy of transgene, according to the Mendelian genetic laws (Tizaoui, Kchouk, 2012; Nghia et al., 2020). The wild-type (WT) plants (Col-0) were used as control.

Plant growth

The seeds were firstly sterilized by ethanol 70% for 1 min and NaOCl 2% (v/v) for 15 min, followed by being rinsed thoroughly with sterilized distilled water prior to aseptically transferred to germination medium (MS medium supplemented with 1% sucrose and 0.8% agar) on petri dishes. These dishes were then kept under cold (4 °C), dark condition for 2 days and transferred to a controlled growth condition (22 °C, 16/8-h day/night period) for seed germination and plant growth (Quach et al., 2014).

Salt-stress treatment

To investigate the effects of salinity on gene
expression, both 14-day-old WT and transgenic Arabidopsis seedlings were transplanted from the germination medium to the plastic trays containing Tribat soil. Normal irrigation was provided for additional 16 days before the salt stress was applied with 200 mM NaCl solution from bottom of the trays (120 mL/2 days/tray) (Jiang et al., 2015). Aerial parts of plants (n=3 biological replicates) were collected at 0 day, 3 days and 7 days after the stress treatment by freezing in liquid nitrogen.

**Total RNA isolation and cDNA synthesis**

Total RNA was extracted from the samples using TRIzol™ Reagent (Thermo Fisher Scientific, USA). RNA quality and quantity were analyzed using spectrophotometry-based method (Thao et al., 2013).

Conversion of the first-stranded complementary DNA (cDNA) was carried out using 1,000 ng total RNA per sample and following the procedure from RevertAid cDNA synthesis kit (Thermo Fisher Scientific, USA).

**Real-time quantitative PCR**

Actin2 was used as the reference gene (Yang et al., 2016). RT-qPCR reactions were prepared in 25-μL-volume, which included 1 μL of cDNA, 12.5 μL SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA) and 0.4 μM of each gene-specific primer. The specific primers for target genes, including Catalase 1 (CAT1); Stromal ascorbate peroxidase 1 (sAPX1); Copper/zinc superoxide dismutase 1 (CSD1); Sodium/hydrogen exchanger 1 (NHX1); and Salt overly sensitive 2 (SOS2), were obtained from previous studies (Table 1). RT-qPCR reactions were carried out using MasterCycler RealPlex4 (Eppendorf, Germany). The PCR conditions were as follow: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Melting curve analysis was performed after the amplification.

**Table 1.** Forward and reverse primer sequences used for RT-qPCR.

| Primer | Sequences | References |
|--------|-----------|-----------|
| Actin2 | 5'-GCACCACCTGAAAGGAAAGTACA-3’<br>5'-CAGTTCCTGGACCTGCCTCATC-3’ | Yang et al., 2016 |
| CAT1   | 5'-TGGGATACGACAGGCAAGAAACG-3’<br>5'-GTTTGGCCTACGTTAAGACGAGT-3’ | Nguyen et al., 2018 |
| sAPX1  | 5'-TGCTAATGCTGGTTCATGACTGGCTT-3’<br>5'-CCACTACCTGTCTGCGCTAGATCTTCC-3’ | Nguyen et al., 2018 |
| CSD1   | 5'-AGACCCGTAGACCTGGAAAGA-3’<br>5'-GCCACACACAGAAGATACAC-3’ | Chen et al., 2013 |
| NHX1   | 5'-GACTCCCTGATGGGAACCCG-3’<br>5'-CCACGTTACCCTCAAGCCTAC-3’ | Li et al., 2015 |
| SOS2   | 5'-GGTGTGAAGAAAATGTAGTCTG-3’<br>5'-GCTACATAGTGGAGTTCCACA-3’ | Zhou et al., 2015 |

**Statistical analysis**

The relative expression of target genes was normalized with the reference gene in the same sample using 2−ΔΔCt method (Livak, Schmittgen, 2001). For target gene expression comparison between the samples, the expression was normalized against the expression of target gene in non-stressed WT, which was used as control sample. The statistical comparison between different genotypes and conditions was conducted via student’s t-test.

**RESULTS AND DISCUSSION**

Plants have developed complex mechanisms to sense and react against environmental stimuli...
(Lamers et al., 2020). Among those, TCS has been identified as one of the most evolutionarily conserved signaling cascades in regulating plant stress response (Grefen, Harter, 2004). Previous studies have discovered the involvement of the soybean TCS member GmRR34 in relation to drought (Le et al., 2011; Thu et al., 2015; Nghia et al., 2020). Therefore, in this study, we further explored the involvement of GmRR34 in another type of osmotic stress, salinity. To do this, five key salinity-responsive genes, including CAT1, sAPX1, CSD1, SOS2 and NHX1, were selected for the expression analysis. A number of studies have revealed that the transgenic plants with better salinity tolerance have enhanced expression of one or more than one of these genes, such as CAT, APX, SOD and SOS in tobacco (Chen et al., 2017), CAT, APX and SOD in bermuda grass (Hu et al., 2012), SOS (Qiu et al., 2004) and NHX (Liu et al., 2010) in Arabidopsis. Examination on expression of these genes under salt stress condition would provide important insights regarding the contribution as well as mechanism by which GmRR34 modulates the stress tolerance in plants.

Expression of antioxidant enzyme-encoding genes

The obtained results in our study showed that the WT and transgenic plants displayed differential expression patterns in all these three studied antioxidant genes (Figure 1). Following the alteration in transcript abundance over the course of salinity treatment for each genotype, it was observed that expression of CAT1, sAPX1 and CSD1 in both transgenic and WT plants was upregulated after 3 days since the stress application. Apart from CSD1, significant increase was observed in CAT1 and sAPX1 for the WT and transgenic plants between the non-stressed and 3-day-stressed conditions (Figure 1). At 7-day salinity stress time point, these genes could not maintain the high expression level status in the WT plants. In fact, at this stage, CAT1, sAPX1 and CSD1 displayed reduction in transcriptional abundance compared with the previous time points of analyses in the WT plants (Figure 1). On the other hand, after 7 days being exposed to the salinity stress, the amount of transcripts of all three antioxidant enzyme-encoding genes in the GmRR34-transgenic Arabidopsis reached their highest levels over the course of treatment, though there was no significant difference between this time point and 3-day time point (Figure 1). However, such decrease in expression of these genes in the WT plants and further increase in their expression in the transgenic plants resulted in a significant difference in expression levels between these two genotypes after being exposed to 7-day salinity treatment. According to the obtained data, CAT1, sAPX1 and CSD1 expressed 2.3-, 2.5- and 3.3-fold more highly, respectively, in the transgenic plants compared with those of the WT plants (Figure 1). Possibly at this stage, the transgenic plants were still able to maintain strong activities of antioxidant enzymes while the WT counterparts could not due to prolonged stress duration.

When plants are subjected to osmotic stress conditions, ROS are built up to significantly higher levels, which cause oxidative damage to plant cell (Wang et al., 2004). In the list of selected genes for expression examination, CAT1 encodes CAT enzyme, sAPX1 encodes peroxidase (POD) enzyme and CSD1 encodes superoxide dismutase (SOD) enzyme. These are antioxidant enzymes that are responsible for the detoxification of excessive ROS (Zhou et al., 2019). In particular, SOD catalyzes the conversion of superoxide-typed ROS into oxygen and hydrogen peroxide (H2O2), while CAT and POD are in turn responsible for hydrogen peroxide (H2O2) removal (Parida, Das, 2005). Therefore, upregulation of these genes might result in increased activity of the corresponding enzymes, which is necessary to protect the plants from salinity stress-induced oxidative damage (Kumar et al., 2018). This means that the transgenic plants might outperform the WT plants in ROS-scavenging capacity with stronger activities of antioxidant enzymes. Previous studies also indicated that enhanced expression levels of these genes under stress conditions such as salinity or drought...
would improve plant detoxification efficiency, which ultimately leads to a better tolerance toward abiotic stress (Habib et al., 2016). Therefore, examining the expression of other ROS-scavenging members such as peroxiredoxin- or glutathione peroxidase-encoding genes is also a potential direction for future study.

![Figure 1](image)

**Figure 1.** Relative expression of antioxidant enzyme-encoding genes (CAT1, sAPX1, and CSD1) in wild-type (WT) and transgenic plants under normal (0-day) condition and salinity treatments for 3 and 7 days. Data represented the means and SEs. Statistically identified differences of stressed samples (3-day and 7-day) compared with control sample (0-day) of the same genotype were indicated by the star symbol. Statistically identified differences between WT and transgenic plants of the same conditions were indicated by linings and the star symbol (*p-value < 0.05).

**Expression of ion-regulator-encoding genes**

Transcriptional profiles of two important ion-regulator-encoding genes, SOS2 and NHX1 were also investigated using RT-qPCR. According to the obtained data, expression level of SOS2 was significantly reduced over the course of stress treatment in the WT plants. In detail, the SOS2 transcript was reduced nearly 44% and 60% after 3-day and 7-day treatment of salinity, respectively, compared with the non-stressed samples (Figure 2). On the other hand, a differential expression pattern for SOS2 was observed in the GmRR34-transgenic plants, which was maintained at a similar level to the non-stressed sample after 3 days of salinity treatment slightly induced upon longer stress exposure (i.e. day seventh) (Figure 2). In addition, only 7-day time point displayed the pronounced difference between the two studied genotypes, whereby the transgenic plants had SOS2 expression level 2.7-fold higher in comparison to that of the WT (Figure 2).

Regarding NHX1, expression pattern of this gene is similar to that of three antioxidant enzyme-encoding genes (Figures 1, 2). After 3 days of salinity treatment, NHX1 expression was up-regulated in both genotypes, though only the transgenic plants displayed significant increase in induction level when comparing between the control and the stressed samples of the same genotype (Figure 2). When the stress duration was extended, the transcriptional level of this sodium ion regulator-encoding gene was slightly reduced in WT, while continuously increased in the transgenic plants (Figure 2). Comparison of the two genotypes under the same condition showed that there was no notable difference in expression level of NHX1 between the WT and GmRR34-transgenic plants at 0-day or 3-day time points (Figure 2). However, transcriptional
amount of NHX1 was significantly higher in the transgenic plants compared with their non-transgenic counterparts after 7 days of stress application (i.e., 2.3-fold higher) (Figure 2).

Under salinity conditions, plants not only suffer dehydration and oxidative stresses but also ionic stress, which negatively affects cellular metabolism. Therefore, scavenging excessive Na\textsuperscript{+} out of cell or storing this ion in specific compartments such as vacuole are strategies that plants can use to prevent Na\textsuperscript{+} disturbance on cellular metabolism (Blumwald, 2000; Chakraborty et al., 2012). Various proteins have been found to be involved in the plant ion regulation, such as the SOSs or the NHXs. In Arabidopsis, SOS family includes three members, which are SOS1, SOS2 and SOS3 (Chakraborty et al., 2012). Previous study found that SOS2 encodes a protein kinase that is required for salinity tolerance in Arabidopsis (Liu et al., 2000). In addition, SOS2 is responsible for activating the activity of SOS1 - the regulator that controls ion homeostasis by excluding the sodium from the apoplast (Chakraborty et al., 2012). Therefore, enhanced expression of SOS2 observed in transgenic plants might indicate better regulation of ionic balance under salinity stress. Another important member, NHX1, is known to lower adverse effects of excessive Na\textsuperscript{+} in the cytosol and maintain osmotic balance by transporting Na\textsuperscript{+} into vacuole in exchange with H\textsuperscript{+} ion (Apse et al., 1999; Blumwald, 2000; Hasegawa et al., 2000). It has been reported that overexpression of NHX1 in Arabidopsis resulted in higher salinity tolerance of the transgenic plants (Liu et al., 2010, Qiu et al., 2004). Furthermore, findings from Qiu et al. (2004) study revealed that activity of vacuolar NHX1 is also regulated by SOS2, which may explain for higher amount of transcript of NHX1 in the transgenic plants in this study (Figure 2). Taken together, these results suggest that the GmRR34-transgenic plants might have better tolerance toward salinity stress by preventing cytosolic Na\textsuperscript{+} accumulation.

![SOS2 and NHX1 expression](image)

**Figure 2.** Relative expression of ion regulator-encoding genes (SOS2 and NHX1) in wild-type (WT) and transgenic plants under normal (0-day) condition and salinity treatments for 3 and 7 days. Data represented the means and SEs. Statistically identified differences of stressed samples (3-day and 7-day) compared with control sample (0-day) of the same genotype were indicated by the star symbol. Statistically identified differences between WT and transgenic plants of the same conditions were indicated by linings and the star symbol (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001).

CONCLUSION

Collectively, this study provided important information regarding the biological functions of GmRR34 in mediating plant response to salinity. The obtained data indicated that GmRR34 might act as a positive regulator, enhancing the plant tolerance toward salinity by at least promoting
activities of antioxidant enzymes to scavenge ROS and preventing cytosolic Na\(^+\) accumulation. Therefore, it is worthwhile to perform GmRR34-related physiological and biochemical studies for comprehensive understanding about its roles as well as deploying GmRR34-based biotechnological application.

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