EVALUATING SALINITY EFFECTS ON TRANSGENIC SOYBEAN HARBORING A CYTOKININ DEHYDROGENASE GENE AT EARLY GROWTH STAGE

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SUMMARY

Soil salinization, along with drought, is a natural threat that has a great impact on agricultural production in many locations throughout the world. The cytokinin oxidases/dehydrogenases (CKXs) play an essential role in determining plant CK levels and several of which have been modulated to improve abiotic stress tolerance. In this study, the aim was to investigate whether there were differential effects of salinity on wild-type (WT) soybean (*Glycine max* L.) plants and a transgenic soybean line harboring *GmCKX13*, which had previously been identified as a responsive gene to osmotic stress. The effects of salt stress were monitored by deploying different concentrations of NaCl. According to the obtained results, the transgenic plants could maintain a better germination rate and radicle growth than the WT plants after a 4-day treatment of 200 mM NaCl. In addition, the transgenic seedlings also displayed a more vigorous shoot and root growth with a larger biomass production under the adverse condition. The analytic data revealed that advantages acquired by the transformed plants at least came from the better capacity to reserve water in the tissue and lower stress damage, as indicated by a lower malondialdehyde content. Taking these findings together, it is suggested that modulating the expression of *CKX13* could contribute to better salt tolerance in soybeans, providing the foundation for conducting more elaborate studies in the future and paving the pathway for the development of elite salinity-tolerant varieties.

Keywords: cytokinin, *Glycine max*, *CKX*, malondialdehyde, salinity stress, seed germination

INTRODUCTION

Glycine max L. Merrill, soybean, is one of the legume species that provides a good source of plant oil and nutritional proteins for human use and is currently the world's most essential oil crop, compared to other crops such as rapeseed and ground pea. According to Nikolić and others (2009), soybean has a high concentration of crucial unsaturated fatty acids such as omega-3, omega-6, as well as omega-9. In addition, soybean seeds are also regarded as an excellent source of vitamins.

Plants all over the world are severely suffering from the high levels of salinity in the environment, which is one of the main abiotic stresses. As reported by the Food and Agriculture Organization (FAO), more than 10% of the cropland has been exposed to salinity problem (FAO, 2021). Fundamentally, mild salt stress causes a negative effect on plant growth, thus agronomic traits and agricultural production, while severe salinity stress can result in the demise of the plants. Salinity can disturb on various stages of legume growth, including germination, early seedling, vegetative and reproductive stages (Mansouri, Kheloufi, 2017). Salinity also reduces nodulation in soybean, which affects nitrogen fixation capacity and ultimately affects yield (Dong *et al.*, 2013; Nitawaki *et al.*, 2020). According to Araújo and others (2015), salinity also reduces the grain quality of legumes.

Specifically, salinity stress has an impact not only on physiological but also biochemical processes in plants. Plants exposed to salt stress will suffer osmotic stress, which is similar to the consequences of drought stress, with reduced water absorption capability from the root organ and promoted plant dehydration. Another attack caused by the stress is intracellular ion homeostasis disorder, which is displayed with the potassium ion (K^+)/sodium ion (Na^+) ratio imbalance and ion toxicity such as Na^+ toxicity (Khan *et al.*, 2015; Isayenkov, Maathuis, 2019; Kumari *et al.*, 2021). Under osmotic stress conditions such as salinity, oxidative stress, which is a secondary stress, is induced due to the accumulation of endogenous reactive oxygen species (ROS) (Song, Wang, 2015; Hasanuzzaman *et al.*, 2021). These active agents tend to attack macromolecules within the cells, resulting in their structural damage and/or the loss of biological functions, thus disruption of cellular activities, and even cell death. For example, ROS-induced lipid peroxidation under salinity conditions can destroy the integrity of the bio-membrane structure, leading to the loss of its function as a biological barrier and a site for enzymatic activities. Furthermore, all of these salt-induced stresses can accelerate the leaf senescence and chlorophyll degradation, leading to the inhibition of photosynthesis and the reduction in yield (Li *et al.*, 2012; Liu *et al.*, 2017; Balti *et al.*, 2021).

The growth and development of a plant are tightly and strongly regulated by phytohormones including cytokinins (CKs). Recent research has also highlighted CK involvement in mediating plant responses to osmotic stress conditions such as salinity (Joshi *et al.*, 2018; Feng *et al.*, 2019; Hyoung *et al.*, 2019). Active CKs are present in different forms, among which N^6-isopentenyl adenine (iP) and *trans*-zeatin (*tZ*) are the most commonly abundant isoprenoid-CKs (Nishiyama *et al.*, 2012). In plants, the metabolism of CK phytohormone is mainly regulated by the CK-synthetic enzymes adenosine phosphate-isopentenyl

transferases (IPTs) and the CK-degrading enzymes oxidases/dehydrogenases (CKXs), which are encoded by *IPT* and *CKX* gene families, respectively (Hai *et al*., 2020). Several studies have shown that appropriate modification of the *CKX* expression and activity can vary the endogenous level of active CKs and contribute to improved plant performance under abiotic stress conditions (Jameson, Song, 2016; Li *et al*., 2019; Chen *et al*., 2020; Hai *et al*., 2020).

As reported by Le and others (2012), soybean *GmCKX13* was a drought-responsive gene, which might play a role in plant adaptation not only under drought but also under salinity conditions, as both belong to osmotic stress and cause similar negative effects to the plant's physiological and biochemical status (Uddin *et al*., 2016). Therefore, in this study, the involvement of *GmCKX13* in salinity response was explored. In terms of the experiments, we focused on evaluating the phenotypic and physiological differences between non-transgenic and transgenic soybean plants harboring *GmCKX13* at the germination and seedling stages following salinity treatment. To interpret the plant performance, we investigated an analysis of the seed germination rate and shoot/root-related characters including malondialdehyde content in the leaf tissue, relative water content in the shoot, length and dry biomass of the shoots and roots under normal and salinity stress conditions.

MATERIALS AND METHODS

Plant materials

The seeds of Williams 82 soybean variety were provided by RIKEN Center (Yokohama, Japan) and used as wild type (WT). This cultivar was also used for the transformation of *PYK10*::*GmCKX13* by *Agrobacterium*, using the service of Iowa State University (USA). The construct had been introduced into the pTF101.1gw1 vector for subsequent bacterial transformation. Mendelian segregation analyses for the ratio of Basta-resistant/Basta-sensitive phenotypes were conducted over three consecutive generations of the transgenic plants to identify the homozygous transgenic line (Tizaoui, Kchouk, 2012). The progenies of the identified homozygous line were used as the experimental materials for this study.

Seed germination assay

The seed germination assay was carried out following previous methods with modification (García-Tejero *et al*., 2015; Hoang *et al*., 2021). In brief, the soybean seeds were first surface-sterilized with 5% sodium hypochlorite (NaClO) for 5 min. Then, the seeds were rinsed three times with sterile distilled water and blotted onto sterile paper. For each petri dish, 10 transgenic soybean seeds and 10 WT soybean seeds were introduced and placed between 2 layers of filter paper. After that, 10 mL sterile liquid, either water (for control) or NaCl solution (at a concentration of 100 mM or 200 mM) was added to the containers. These petri dishes were incubated under laboratory conditions (25°C, dark condition) for 4 days before the germination rate was recorded.

For recording the final germination percentage (FGP), the seeds with a radicle length of a minimal 2 mm were regarded as germinated (Wijewardana *et al*., 2019). In addition, radicle length of successfully germinated seeds was also measured. For each genotype per treatment, four experimental replications (i.e., four petri dishes) were prepared.

Plant growth for analyses at the vegetative stage

After germinating the transgenic and the WT soybean seeds on soil for seven days, these seedlings were transferred to hydroponic tanks (six seedlings of WT and six seedlings of the transgenic plants per container) containing 10 L of commercial hydroponic nutrient solution of Nong Hoa Xanh Ltd. Company (Vietnam) (Kim *et al.*, 2018). The plants were allowed to grow under net-house conditions (28–33°C, 60–70% humidity, and natural photoperiod). After 15 days growing in a hydroponics-based system, NaCl solution at a concentration of 50 or 100 mM was applied. Plants without salt treatment was also included and used as control (6 tanks per treatment). The duration for stress application was 12 days.

Analysis of shoot and root characters

To assess the shoot- and root-related traits, the plants were removed from the hydroponic tanks carefully at the end of the stress treatment. The roots and shoots of individual plants were dissected before their length and dry biomass were recorded (n ≥ 10 per genotype per treatment).

In addition, the relative water content (RWC) in the shoot tissue was also determined. To do this, following the length measurement, the fresh weight (FW) of the shoots was recorded. Next, the shoot samples were soaked in distilled water overnight and the turgid weight (TW) was measured. Finally, these shoots were dried at 65°C for 48 h before the dry weight (DW) was documented. The RWC was calculated using the formula: RWC (%) = [(FW−DW)/ (TW− DW)] × 100 (Thu *et al.*, 2014).

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Analysis of malondialdehyde (MDA) content

To evaluate the degree of membrane lipid peroxidation in the non-stressed and stressed plants, malondialdehyde (MDA) content was measured using a thiobarbituric acid (TBA) assay (Demirel *et al.*, 2020; Senthilkumar *et al.*, 2021). In brief, 0.2 g of the leaf tissue powder was ground in 1 ml of 0.1% trichloroacetic acid (TCA). After that, the homogenized samples were centrifuged at 12,000 rpm, 4°C for 15 min. Next, the supernatant was transferred to a new tube containing 20% TCA with 0.5% TBA in a ratio of 9 supernatant:10 reaction solution (v/v) and mixed well. The mixture was heated in a water bath at 95°C for 15 min, then rapidly cooled in an ice bath for 10 min, followed by centrifugation at 10,000 rpm, 4°C for 5 min. Finally, the optical density of the supernatant was measured at 532 nm and 600 nm wavelengths. The reaction solution was used as blank. The MDA was calculated using the formula: MDA (μmol/g FW)=[(A$_{532}$-A$_{600}$/155] x 10^3 x dilution factor x (1/tissue weight g) (Demirel *et al.*, 2020). For this assay, the leaf samples were collected at 0, 3rd, 7th, and 12th day since stress application, with three replicates.

Statistical analysis

The data in all experiments were analyzed using ANOVA by GraphPad Prism (version 9.1, Inc. La Jolla, USA) and Tukey post-hoc test for identification of any difference among the treatments for each genotype, and the *t*-test for identification of any difference between two studied genotypes under the same condition, with the setting of p-value < 0.05.

RESULTS AND DISCUSSION

All plant tissues contain CKs that regulate various developmental processes, including cell division, apical dominance, leaf senescence, as well as root and shoot differentiation (Trifunović-Momčilov *et al.*, 2020). It is well known that CK can modulate the osmotic stress tolerance of plants in both positive and negative manners, as increasing or reducing CK contents could result in increased plant tolerance such as towards drought, heat and salinity (Lubovská *et al.*, 2014; Pospíšilová *et al.*, 2016; Hai *et al.*, 2020). For the latter strategy, lowering endogenous CK levels can be achieved by promoting the activities of CK degradation-related enzymes, the CKXs (Lubovská *et al.*, 2014; Pospíšilová *et al.*, 2016; Li *et al.*, 2019). In addition, as drought and salinity cause similar negative effects imposed on plants as well as trigger similar plant responses (Khandal *et al.*, 2020; Ma *et al.*, 2020), *CKX* manipulation can also confer the plants improved tolerance towards either or both stressors (Nishiyama *et al.*, 2011; Li *et al.*, 2019). In this study, we were interested in exploring the attributes of *GmCK13*, which was previously identified as a drought-responsive gene (Le *et al.*, 2012), in plant responses to salinity.

Effects of salt stress on seed germination and radicle elongation

Seed germination and post-germinative development are important in the early stages of a plant's cycle. Favorable environmental conditions such as ideal temperature and light promote germination, whereas other factors such as high salinity can delay or inhibit this process (Shuai *et al.*, 2017; Uçarlı, 2020). This adverse outcome was also observed in our germination assay, with a negative correlation between the germination rate and the applied salt concentration (Figure 1A). Upon 100 mM NaCl treatment, the germination rates recorded after 4-day incubation were 95% for the transgenic plants and 87.5% for the WT, equivalent to a reduction rate of 5% and 12.5%, respectively, in comparison with the germination rates under non-stressed conditions. At a higher applied salt concentration (200 mM NaCl treatment), the germination rates in both genotypes were considerably decreased compared to the control counterparts, particularly at a much lower rate in the WT group. In detail, under this condition, the WT seeds had an average germination rate of 60% whereas the transgenic plants could maintain the successful germination at a rate of 77.5%.

In addition, the length of emerging radicle from germinated seeds was also examined. Generally, there was a significant decrease in the radicle length of the germinated seeds as the higher salt condition was applied (Figure 1B). On the fourth day since incubation, the average radicle length for the control treatment was 3.085 cm for the WT and 3.345 cm for the transgenic plants. The average radicle lengths under the low salt treatment (100 mM NaCl) across the two genotypes were reduced by more than 50% (i.e. 1.271 cm for the WT and 1.395 cm for the transformant). With the high salt treatment (200 mM NaCl), the growth of the radicles was substantially restricted, with an average radicle lengths equivalent to approximately a sixth and a fifth of the corresponding lengths of the WT and transgenic plants under the non-stressed conditions, respectively. However, the statistical analysis revealed that under this harsh condition, the transgenic plants had

significantly longer radicles compared with the WT (Figure 1B).

According to the study of Essa (2002), soybean germination was inhibited by salinity, and soybean seeds that could germinate on saline soil displayed growth and biomass penalties as well as a possible reduction in the weight of seeds produced by these plants. The mechanisms of soybean germination and growth inhibition by salinity are not completely understood, especially for the genetic mechanism (Zhang *et al.*, 2014). Under the salinity condition, it has been known that the stress maintains the seed dormancy by increasing the biosynthesis of abscisic acid yet decreasing the biosynthesis of gibberellic acid (Shu *et al.*, 2017). In addition, the multifaceted regulatory roles of CK on seed germination were also observed. Application of exogenous CKs or increasing CK levels could result a higher seed germination rate (Nikolić *et al.*, 2006; Wang *et al.*, 2011). However, it has been reported that disrupting the CK signaling could also promote a higher germination rate (Riefler *et al.*, 2006). The hypothesis to explain this is the antagonistic interaction between the CK- and gibberellin-signaling pathways. When the former is suppressed, the latter is enhanced thus stimulating the seed germination (Riefler *et al.*, 2006).
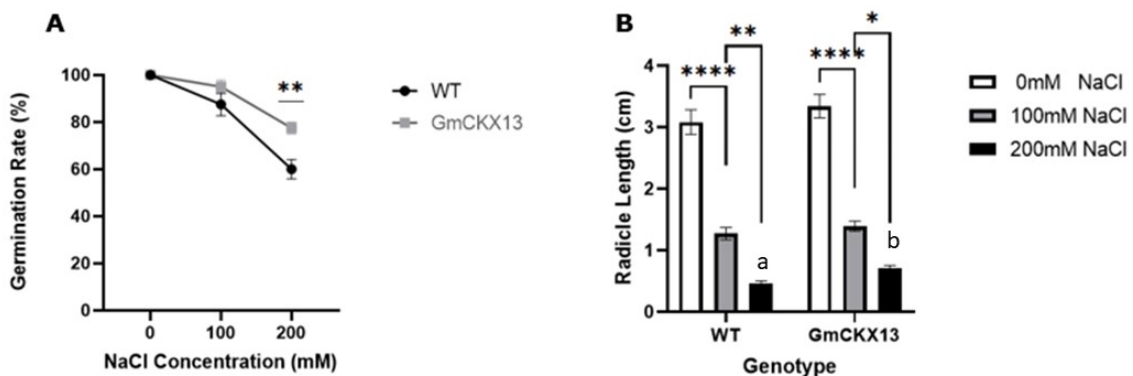
Figure 1. Examination of salinity effects on seed germination of wild-type (WT) and transgenic plants (GmCKX13). (A) The average germination rates. (B) The average radicle length. Values are means ± SE. The asterisk stands for the significant difference under different treatments for the same genotype, with *p < 0.05, **p < 0.01 and ****p < 0.0001. The letter symbol indicates the significant difference between the genotypes under the same treatment (p < 0.05).

Effects of salt stress on growth characteristics

We further examined the salt effects on plant growth at the vegetative stage. As expected, the stress significantly depressed the growth of the two genotypes (Figure 2). Although there was no distinct difference in shoot lengths between the two studied genotypes, the salinity caused a higher reduction rate in the shoot length in the WT (by 12%) than in the transgenic plants (by 9%) between any two adjacent concentrations of NaCl application (0 mM versus 50 mM and 50 mM versus 100 mM) (Figure 2A). A similar result was also observed for the root length parameter, with the average reduction rates in the WT and the *GmCKX13*-transgenic plants were 12-13% and 6-8%, respectively (Figure 2C).

Regarding the tissue biomass, the salt-induced penalties were more severe. Under normal growth conditions, both genotypes shared similar average DWs of the shoot and root tissues (approximately 0.7 g) (Figures 2B, 2D). Following the 100 mM NaCl treatment, the transgenic plants could maintain significantly higher shoot DW than their WT counterparts. With the 200 mM NaCl application, differential values in biomass were recorded for both shoot and root tissues between the two genotypes. As shown in Figures 2B and 2D, the average shoot and root DWs of the WT were *ca.* 0.37 g whereas the corresponding values in the transgenic plants were 0.52 g and 0.43 g, respectively. This means that if compared to the controls, the WT plants had a higher reduction rate in growth (by 47-52%) compared to the transformants (by 31-38%).
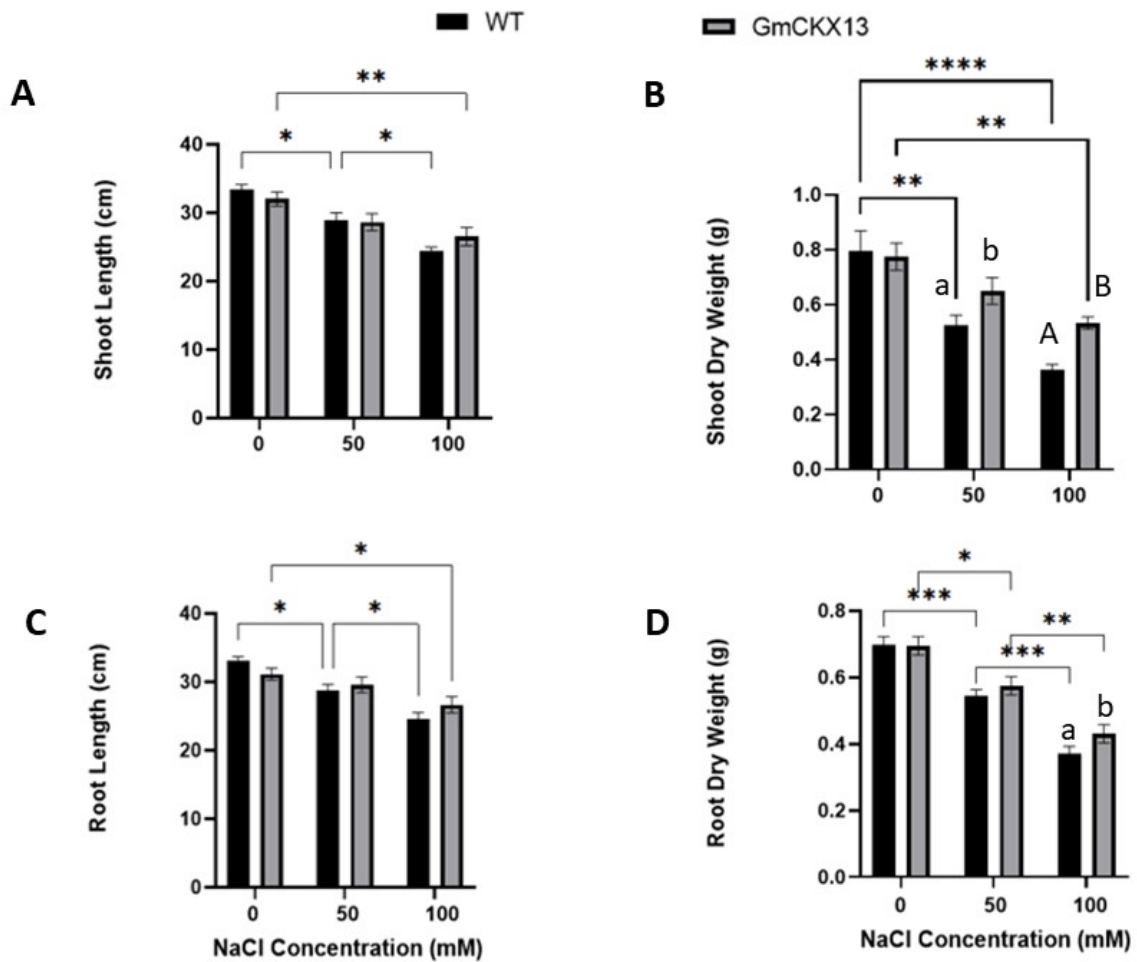

Figure 2. Shoot- and root-related characters of wild-type (WT) and transgenic (GmCKX13) soybean under different salinity conditions. (A) Average shoot lengths. (B) Average shoot dry weights. (C) Average root lengths. (D) Average root dry weights. Values are means ± SE. The asterisk stands for the significant difference under different treatments for the same genotype, with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. The letter symbol indicates the significant difference between the genotypes under the same treatment (small letters $p < 0.05$; capital letters $p < 0.0001$).

Soybeans have different water requirements during the growing season, and they can be vulnerable to drought or salinity during the vegetative as well as the flowering stages. As CKs play a vital role in root and shoot growth, they are also involved in the expression of developmental and architectural traits necessary to tolerate water-limiting conditions (White, Kirkegaard, 2010). At the organ level, roots are very important for plant growth and development, which are mainly responsible for water and nutrient absorption (Nishiyama *et al.*, 2011). Laplaze and others (2007) found that CKs reduced primary root growth and lateral root density in *Arabidopsis thaliana*. Therefore, when the levels of endogenous CK in roots decrease, for example by enhancing the root-specific expression of *CKX* genes, the increased root growth and branching can be observed (Werner *et al.*, 2010). In the present study, although there was no difference in the average primary root lengths of the two genotypes under non-stressed and stressed conditions, the transgenic plants displayed a higher root biomass upon 100 mM NaCl application. This was because they had more lateral roots than the WT (Figures 2C, 2D). The better root characters might support the better growth of the shoots, as displayed in the transgenic plants (Figures 2A, 2B). In addition, as CK is required for shoot growth via stimulating cell division and shoot proliferation but suppresses the root growth (Kieber, Schaller, 2018), we used the root-specific promoter (*Arabidopsis PYK10*) to drive the expression of the transgene *GmCKX13*, to enlarge the root system and minimize the negative effects on the growth of aerial organs.

Effects of salinity on shoot relative water content

Figure 3 shows that applying salt stress at

the vegetative growth stage reduced the RWC in soybeans and as the salt stress level increased, the decrease in this parameter became more dramatic. According to the obtained results, while the normal tissues had around 80% RWC, the RWCs in the salt-treated plant with 50 and 100 mM NaCl were 65.62% and 59.76% for WT, and 69.70% and 65.84% for *GmCKX13*-transgenic plants, respectively. However, comparing the RWCs between the two genotypes under the same salt treatment, the latter had significantly maintained a higher level of water content in the shoot tissues.
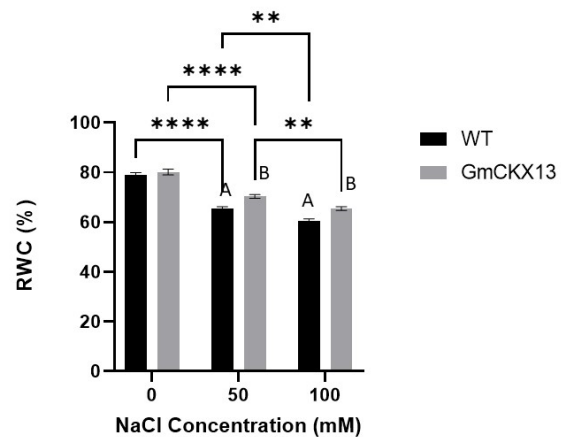
Figure 3. Effects of salinity on the shoot relative water content (RWC) of wild-type (WT) and transgenic (GmCKX13) plants under different salt stress conditions at the vegetative growth stage. The salt treatment had been applied for 12 days before the values were recorded. Values are means ± SE. The asterisk stands for the significant difference above the data points, with **p < 0.01 and ****p < 0.0001. The letter symbol indicates the significant difference between the genotypes under the same treatment, with p < 0.01.

Similar to drought stress, salinity also causes plant dehydration. Therefore, better water retention ability in the tissues is considered an advantage for the plant to survive under harsh conditions (Suriya-arunroj *et al.*, 2004; Verslues *et al.*, 2006;

Ievinish, 2023). Previous studies also indicated a positive correlation between water reservation and plant tolerance to the challenged osmotic stress (Lubovská *et al.*, 2014; Liu *et al.*, 2020; Chuong *et al.*, 2021). Various factors have been known to contribute to the RWC in the tissues, including water absorption capacity by the root system, concentrations of endogenous osmotic regulators and stomatal conductance. Previously, a transgenic study in tomatoes constitutively overexpressing *Arabidopsis CKX3* had higher RWC in the leaf organ, and reduced transpiration rate, partially due to the decrease in stomatal density (Farber *et al.*, 2016).

Effects of salinity on lipid peroxidation in soybean leaves

Under high salinity conditions, plant growth and development are adversely affected by the disrupted homeostasis. One of the main causes for this is the accumulation of endogenous ROS and their interference with the structure and functions of the cellular macromolecules (Hasanuzzaman *et al.*, 2021). Therefore, the lipid peroxidation of the cell membrane in the leaf tissues, as a result of ROS attack to the lipid molecules on the membrane under salinity conditions was evaluated based on the MDA content, to assess the degree of membrane integrity and the stress-induced injury. According to the findings, both WT and transgenic plants under control conditions displayed a very low MDA level (Figure 4). When being challenged with the salt stress, although the MDA contents were increased over the course of the stress treatment and along with the increased NaCl concentrations, there was a greater rise in the WT than those in the transgenic plants. In detail, compared to the control treatment, the MDA content rapidly increased by 39-fold in the WT and 35-fold in the transgenic plants after 7-day treatment, and 43-fold in the WT and 39-fold in the transgenic group after 12-day treatments with 50 mM NaCl (Figure 4A).
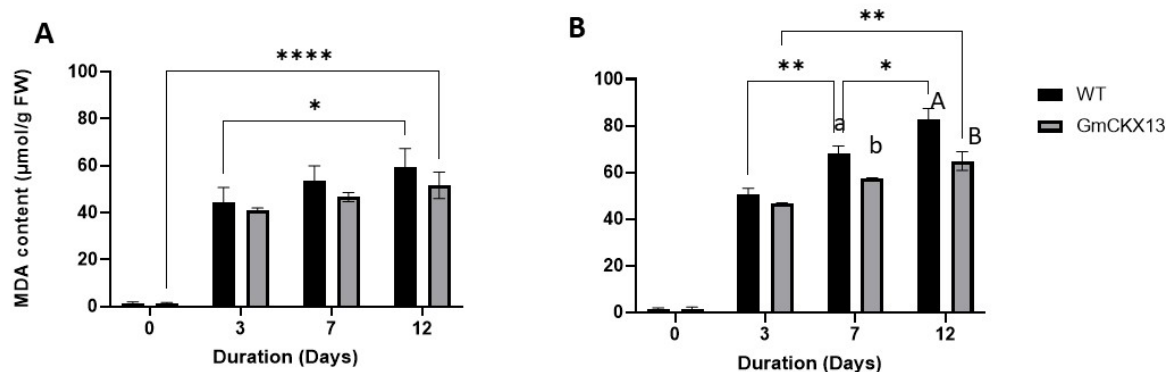
Figure 4. Analysis of salt stress-induced oxidative stress, based on the malondialdehyde (MDA) content to evaluate the lipid peroxidation in the leaf tissue of wild-type (WT) and transgenic (GmCKX13) plants. (A) MDA content analysis following 50 mM NaCl treatment. (B) MDA content analysis following 100 mM NaCl treatment. Values are means ± SE. The asterisk stands for the significant difference under different treatments for the same genotype, with *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. The letter symbol indicates the significant difference between the genotypes under the same treatment (small letters $p \leq 0.05$; capital letters $p \leq 0.0001$).

Under 100 mM NaCl condition, differential MDA contents between the two examined genotypes were observed (Figure 4B). With a 7-day duration treatment, the WT plants had an MDA level which was 20% higher than the average level quantified from the transgenic samples. Meanwhile, the difference in the MDA contents following 12-day salt exposure between these two groups was even larger, i.e. by 30%. Therefore, these results indicate a possibly less severe oxidative stress imposed on the transgenic plants compared to their counterparts.

Previously, Li and others (2019) reported that overexpression of the *MsCKX* gene from *Medicago sativa* enhanced the activity of ROS-scavenging antioxidant enzymes in transgenic *Arabidopsis* plants. Therefore, analysis of major ROS contents such as hydrogen peroxide (H_2O_2) and $O_2^{\cdot}$ radicals as well as antioxidant enzyme activities in future research would provide more supporting evidence for this finding. Taken all the results together, it is suggested that GmCKX13 might act as a positive component in mediating the plant resistance to salinity.

CONCLUSION

The findings from this research suggest a beneficial contribution of GmCKX13 activity in plant response to salinity. Under the stress conditions, the *GmCKX13*-transgenic soybean plants displayed a better germination rate, more vigorous growth, more efficient water conservation, and a less damaged cellular membrane. To reinforce the statement, other biochemical and molecular analyses should be conducted in future investigations to fully reveal if the biological functions of this CK-metabolic gene can contribute to enhance the salinity tolerance, before its potential application for crop improvement can be evaluated and deployed.

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