

## EVALUATING PHOTOSYNTHETIC PERFORMANCE OF TRANSGENIC SOYBEAN HARBORING *CKX13* UNDER SALINITY CONDITIONS

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### SUMMARY

Cytokinins (CKs) are considered one of the critical phytohormones with an important role in plant response to abiotic stresses. One of the key enzymes in regulating CK levels is CK oxidase/dehydrogenase (CKX), which catalyzes the irreversible CK degradation. Although *CKX* gene members in soybean (*Glycine max*) have been identified, their importance in plant tolerance against salinity has not yet been fully elucidated. In this study, *GmCKX13*, a gene with upregulated expression in various tissues of soybean at various stages under water deficit condition as shown in previous investigation, was selected for exploring its role in relation to photosynthetic performance under salt stress conditions. The results showed that the transgenic soybean overexpressing *GmCKX13* could maintain a better assimilation rate compared to their wild-type (WT) counterparts under the salinity condition. This could be explained by the higher chlorophyll content and the less damage caused by the stress in the photosynthetic machinery of the transgenic plants. According to the analyses, the transformers displayed higher values of maximal photosystem II (PSII) photochemical efficiency ( $F_v/F_m$ ), maximal quantum yield of primary PSII photochemistry ( $\phi_{Po}$ ), quantum yield for electron transport ( $\phi_{Eo}$ ) and efficiency for electron being transferred from  $QA^-$  to plastoquinone ( $\psi_{Eo}$ ). The evaluation for the overall photosynthetic performance using performance index in absorption basis ( $PI_{Abs}$ ) also supported the differential photosynthesis potential between the two genotypes. Collectively, these findings suggested that appropriate *GmCKX13* modulation could enhance photosynthetic adaptation to salt-stressed conditions in soybeans.

**Keywords:** cytokinin, cytokinin oxidase/dehydrogenase, *Glycine max*, photosynthesis, salinity stress

### INTRODUCTION

Soil salinity is a worldwide environmental issue that can critically influence plant and crop growth (Zörb *et al.*, 2018). This threat has been rapidly spreading to at least one-third of the

irrigated and arable land in the world (He *et al.*, 2015). Salinity increases concentrations of particular ions in soil such as  $Na^+$  and  $Cl^-$ , leading to osmotic stress, specific ion toxicity, and nutrient deficiencies in plants, thereby affecting all major processes such as growth, nodulation,

protein synthesis, lipid metabolism, photosynthesis, seed quality and quantity (Daba, Qureshi, 2021; Ilangumaran *et al.*, 2021). According to Guo and colleagues (2015), high salt concentrations significantly interfere with the optimal concentrations of cellular cations such as  $K^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$ , which are crucial in plant photosynthesis. The salt stress can further inhibit photosynthesis by damaging photosystem (PS), decreasing electron transport activity as well as content and activity of Rubisco, which results in yield loss (Gong *et al.*, 2018; Najjar *et al.*, 2019). Like many other crops with low salinity resistance, soybean (*Glycine max*) production can be dramatically reduced under this adverse condition (Ilangumaran *et al.*, 2021). Meanwhile, soybean is one of the leading commercial oilseed species among crop plants, accounting for almost 29% of global oilseed production (Staniak *et al.*, 2023). Processed soybeans, both for human consumption and animal feed, are the most abundant plant source of vegetable oil and proteins (Rahman *et al.*, 2022). Therefore, improvement of salinity stress tolerance in plants and soybean, in particular, has become an indispensable mission to secure agricultural productivity and sustainability (Zhu, 2016).

Cytokinin (CK) is a multifaceted phytohormone that regulates many important plant developmental processes such as shoot and root architecture (Gu *et al.*, 2018), germination (Mok, 2019), and fruit development (Gan *et al.*, 2022). In recent years, the role of CK-related genes in stress-associated cellular response regulation and adaptation, as well as targeted control of CKs metabolism are under intensive investigations to gain more knowledge and understanding, which might subsequently be applied for trait improvement. CK metabolic pathways, in plants, are stably regulated by two major groups of enzymes - adenosine phosphate-isopentenyl transferases (IPTs) and CK oxidases/dehydrogenases (CKXs) (Werner, Schmülling, 2009; Hoyerová, Hošek, 2020). Many studies have supported that irreversible CK degradation, which is catalyzed by the

CKXs, has a significant impact on the regulatory activities of this phytohormone (Mandal *et al.*, 2022). The evolutionary development of CK-catabolizing gene families and the individual properties of their members indicate an essential role in the fine-tuned control of catabolism, which ensures the precise regulation of various CK active forms (Khuman *et al.*, 2022).

Even though there have been a few studies exploring the resistance of transgenic plants overexpressing *CKX* in response to salinity stress (Vankova *et al.*, 2011; Avalbaev *et al.*, 2016; Li *et al.*, 2019), there are no publications on the evaluation of physiological parameters and photosynthetic activity in *CKX*-transgenic soybean under salt stress. Meanwhile, there is a close relationship between photosynthetic activity and productivity. Therefore, in this study, a CK metabolic gene from soybean (*GmCKX13*), which was previously identified as a highly drought-inducible gene (Le *et al.*, 2012), was chosen to evaluate its mediating role to photosynthetic performance in relation to salinity stress. To do this, the carbon assimilation, the gas exchange rate, chlorophyll content, and chlorophyll fluorescence parameters were examined and compared between a transgenic soybean line overexpressing *GmCKX13* and the wild-type plants under normal and salt stress conditions. The obtained results are expected to enhance understanding on the roles of *GmCKX13* in plant response to salinity as well as consider its potential application in development of salt-tolerant crops.

## MATERIALS AND METHODS

### Plant materials and growth conditions

The wild-type (WT) soybean (W82) was supplied by RIKEN Center (Japan). The transgenic soybean seeds harboring *PYK10::GmCKX13* construct were generated by Iowa State University (USA) service, using *Agrobacterium*-mediated transformation method and also W82 as the background genotype for transformation. Following the track of heredity and segregation based on Mendelian genetics

over consecutive generations (Tizaoui, Kchouk, 2012), a transgenic line that carried a single copy of transgene in homogenous form was successfully identified and used as experimental materials of this research.

For germination, the seeds were soaked in water for 30 min at 37 °C, then sown on plastic trays containing Tribat soil. The 5-day-germinated seedlings were subsequently transferred to Styrofoam boxes (36x36x38 cm) for hydroponic cultivation following previous method (Shelke *et al.*, 2017), with modification. Each box contained a solution of 10 L tap water and 50 mL Hydro optimal hydroponic solution from Nong Hoa Xanh Ltd. Company (Vietnam) as the alternative to Hoagland hydroponic solution. All plants were cultivated inside a net-house with temperatures ranging 28-33 °C, relative humidity 60-70 %, and natural photoperiod of 12-h light/ 12-h dark. Soybean seedlings were grown until they were available for salinity application.

### Salinity treatment

The salinity treatment experiment was carried out when the plants were 18 days old. Uniformly grown seedlings of the two genotypes were selected and thereafter divided them into five groups treated with either 0, 50, 100, 150 or 200 mM NaCl. Four WT and four transgenic plants were used for the analysis in each treatment. For salt-treated groups with high NaCl concentration, the plants were challenged with NaCl at the 50 mM NaCl increment every consecutive day until reaching the final concentration (Colomba *et al.*, 2013; Shelke *et al.*, 2017). The stress conditions were maintained for a week.

### Measurement of gas exchange indices

Gas exchange measurements were performed on the attached leaves with a portable photosynthesis system (LI-6800, LI-COR, USA). The measurement was performed in the morning and by using the most recently fully expanded trifoliate leaves. Measurement conditions within the leaf chamber were set at

temperature 27 °C, relative humidity 70 %, light intensity 1000  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , and  $\text{CO}_2$  concentration of 396.4  $\mu\text{mol per mol air}$  (Sheteiwiy *et al.*, 2021). The parameters of interest included assimilation rate ( $A$ ) and stomatal conductance ( $g_s$ ).

### Measurement of chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed on the attached leaves with a portable chlorophyll fluorometer (Handy PEA<sup>+</sup>, Hansatech Instruments, UK) on four plants per treatment per genotype. After darkening a leaf area for 30 min, the minimum ( $F_o$ ) and maximum ( $F_m$ ) fluorescence intensities were measured by the instrument. From this data, the maximum quantum efficiency of PSII was estimated based on the ratio of variable ( $F_v$ ) fluorescence over the  $F_m$  (Schreiber, 1994), whereby the  $F_v$  value was worked out by subtracting the  $F_m$  to the  $F_o$ . Other parameters including the maximal quantum yield of primary PSII photochemistry ( $\phi_{Po}$ ), quantum yield for electron transport ( $\phi_{Eo}$ ), efficiency of electron moving further than  $\text{QA}^-$  ( $\psi_{Eo}$ ) and performance index in absorption basis ( $PI_{ABS}$ ) were also obtained from Handy PEA<sup>+</sup> measurement (Kumar *et al.*, 2020).

### Measurement of chlorophyll content

The relative leaf chlorophyll contents in the studied soybean genotypes were measured using a chlorophyll meter (SPAD (Soil Plant Analysis Development) meter-502, Konica Minolta, Japan) (Do *et al.*, 2016). Four biological replicates were used for the measurement.

### Data analysis

Differences among the treatments as well as between the genotypes were tested using SPSS statistical software (version 11; SPSS Inc., USA). For multiple comparisons, the data first were performed by one-way analysis of variance (ANOVA) to check the equality of variance (Levene-test), then Tukey's multiple comparison tests were used to determine the significant difference ( $p$ -value < 0.05) of means. The data values were presented as mean  $\pm$  standard error (SE).

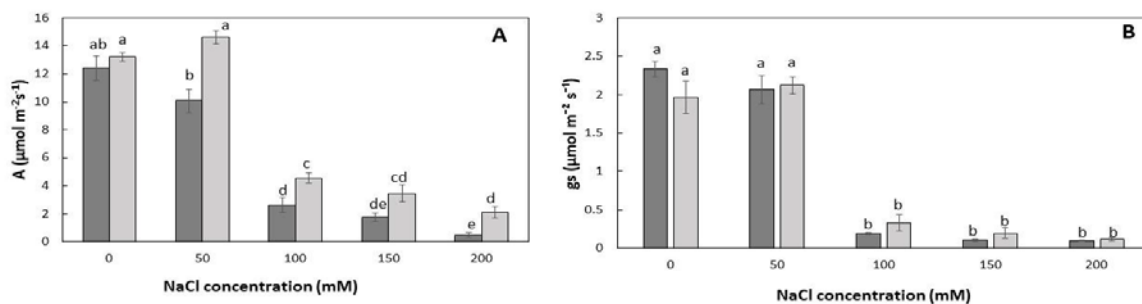
## RESULTS

**The transgenic plants displayed better carbon fixation under the stress condition**

Among the photosynthetic parameters, carbon assimilation is an important value highlighting the overall photosynthetic efficiency. Therefore, we evaluated the *A* values, which were measured by LI-6800 under the standard conditions for other parameters regarding light, temperature, and ambient CO<sub>2</sub> concentration. As shown in Figure 1A, a harsher salinity stress resulted in a more substantial decrease in carbon assimilation rates in both studied genotypes. Noticeably, under 50 mM NaCl application, although there was a decrease in carbon assimilation in the WT plants by approximately 20 % compared to the corresponding value in the WT plants grown normally, this trend was not observed in the transgenic plants. In fact, the assimilation rate in

the latter was slightly enhanced by 10 % under this mild stress condition. With the highest concentration of NaCl used in the assay, the *A* values dropped by almost 96 % and 84 % in the WT and the transformants, respectively (Figure 1A). Statistical analyses revealed that the transgenic plants could maintain a significantly higher carbon fixation rate under such extreme conditions compared with the WT group.

As the carbon assimilation mainly depends on available CO<sub>2</sub> in the leaf tissue, we next examined stomatal conductance activity. The findings also indicated a substantial decrease in stomatal conductance in response to degree of salinity severity. Introduction of NaCl at 100 mM or higher could profoundly inhibit the gas exchange with the reduction rate by 83 to 96 % (Figure 1B). However, under the same treatment condition, there was no obvious difference in stomatal conductance between the WT and *GmCKX13*-transgenic plants.



**Figure 1.** Effect of 7-day NaCl treatments on assimilation rate (*A*) (A) and stomatal conductance (*g<sub>s</sub>*) (B) in leaves of wild-type (dark bar) and *GmCKX13*-transgenic (light bar) soybeans. Vertical bars represent mean ( $n=4$ )  $\pm$  SE. Different letters (a, b, c, ...) indicate significant differences between means at  $p$ -value < 0.05.

**The transgenic plants had higher chlorophyll fluorescence and chlorophyll content**

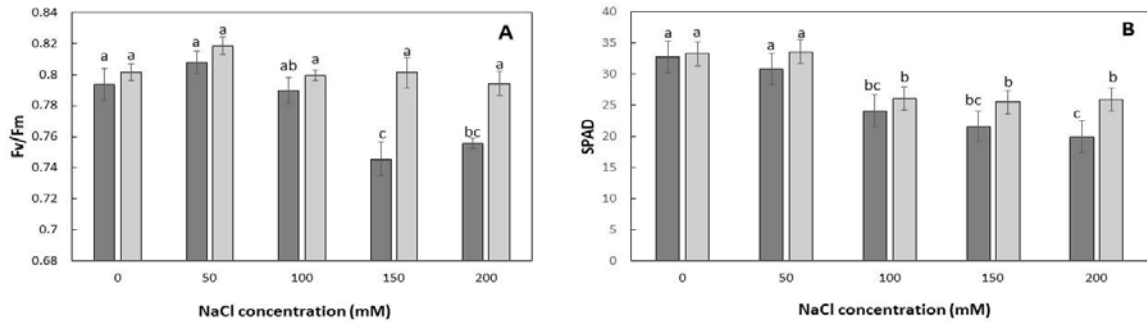
In the light-dependent activities of photosynthesis, the efficient electron transport chain used for subsequent synthesis of ATP and NADPH is the key attribute for a high photosynthetic performance (He *et al.*, 2015; Cardona *et al.*, 2018). In addition, the photochemical quenching for such activity depends on the function of photosystems. Therefore, when there is damage in the structure

of a PS, the electron transport chain activity could be adversely influenced. In this study, to assess the impact of salinity on photosynthetic machinery, we analyzed the chlorophyll fluorescence based on *F<sub>v</sub>/F<sub>m</sub>* ratio. According to Figure 2A, these values remained relatively constant at *ca.* 0.8 in the *GmCKX13*-transgenic soybeans, while there was a decrease in this ratio in the WT group under different salt concentrations. Upon treatments of high salt concentrations of 150 and 200 mM, the *F<sub>v</sub>/F<sub>m</sub>* ratios in the non-transgenic plants were reduced

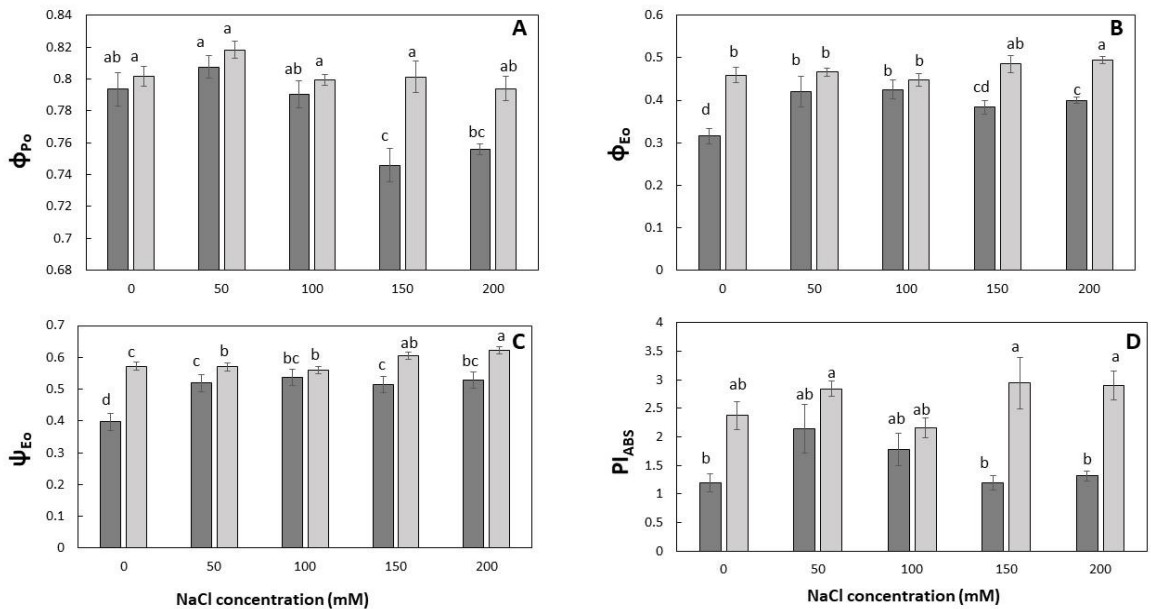
to approximately 0.75. Such differential effects of salinity on these two genotypes, therefore, resulted in a significantly higher  $Fv/Fm$  in the *GmCKX13*-transgenic plants compared to their counterparts under the high salt conditions.

Leaf chlorophyll content of both genotypes was also affected by salinity stress (Figure 2B). However, the WT plants showed greater reduction in the average pigment content than the

*GmCKX13*-transgenic plants. When comparing the relative chlorophyll contents indicated by SPAD meter in the 200 mM NaCl-treated group versus the control group, these values were considerably reduced by 39 % and 22 % in the WT and transgenic soybeans, respectively. This means that the chlorophyll contents of the transgenic plants were significantly higher than those of WT under 200 mM salt stress conditions.



**Figure 2.** Effect of 7-day NaCl treatments on maximal photosystem II photochemical efficiency ( $Fv/Fm$ ) (A) and chlorophyll content (SPAD) measured by SPAD meter (B) in leaves of wild-type (dark bar) and *GmCKX13*-transgenic (light bar) soybeans. Vertical bars represent mean (n=4) ± SE. Different letters (a, b, c, ...) indicate significant differences between means at p-value < 0.05.



**Figure 3.** Effect of seven-day NaCl treatments on maximal quantum yield of primary photosystem II photochemistry ( $\phi_{Po}$ ) (A), quantum yield for electron transport ( $\phi_{Eo}$ ) (B), efficiency that an electron moves further than  $Q_A^-$  ( $\psi_{Eo}$ ) (C) and performance index in absorption basis ( $PI_{ABS}$ ) (D) in leaves of wild-type (dark bar) and *GmCKX13*-transgenic (light bar) soybeans. Vertical bars represent mean (n=4) ± SE. Different letters (a, b, c, ...) indicate significant differences between means at p-value < 0.05.

### Other parameters also demonstrated better photosynthetic performance in the transgenic plants

Performance of PSII was further assessed using the flux ratio parameters  $\phi_{Po}$ ,  $\phi_{Eo}$ , and  $\psi_{Eo}$ . In *GmCKX13*-transgenic soybeans, the  $\phi_{Po}$  values remained relatively constant, while the  $\phi_{Eo}$  and  $\psi_{Eo}$  increased by 8 % and 16 %, respectively, under 200 mM NaCl treatments in comparison to the control (Figures 3A, 3B, 3C). Under the same condition, the overexpressor displayed higher  $\phi_{Po}$ ,  $\phi_{Eo}$ , and  $\psi_{Eo}$  than those measured in the WT counterparts, especially with significant difference under applied NaCl conditions of 150 mM and 200 mM and for the maximal quantum yield PSII ( $\phi_{Po}$ ) values.

With the performance index  $PI_{ABS}$ , the WT plants showed substantially lower values than the transgenic soybeans following high salinity stress exposure (Figure 3D). Upon the treatment of 150 or 200 mM NaCl, the transgenic plants had  $PI_{ABS}$  values that were two-fold higher than the corresponding values found in the WT. Looking at the change in  $PI_{ABS}$  values of individual genotypes exposed to NaCl from low to high concentrations, it was recognized that while the WT plants had slightly decreased  $PI_{ABS}$ , whereas the *GmCKX13*-transgenic plants showed slightly increased performance index.

### DISCUSSION

Photosynthesis is a complex process and highly sensitive to abiotic stress factors, which in turn reduces crop growth and productivity (Shao *et al.*, 2014; Najar *et al.*, 2019; Staniak *et al.*, 2023). Although reduction of the photosynthetic performance under salinity condition has been studied, the underlying mechanism is still not fully understood. Long-term exposure to high salinity levels may cause the plant's photosynthetic activity to be inhibited by early senescence of older leaves, damage in PS structure and thus becoming less functional, degradation of chlorophyll pigments, and closure of stomatal aperture followed by a reduction in

intracellular CO<sub>2</sub> availability for carbon fixation (He *et al.*, 2015; Hnilickova *et al.*, 2021).

The efficiency of photosynthesis can be evaluated by examining the degree of carbon assimilation, which contributes to biomass accumulation and plant growth. Calvin cycle and carbon assimilation can progress depending on various factors such as availability of intracellular CO<sub>2</sub>, which relates to stomatal conductance activity, pigment content, Rubisco activities and outputs of light-dependent reactions. Kao *et al.*, (2003) compared the responses of photosynthetic gas exchange of three soybean cultivars and found that assimilation rate, stomatal conductance, transpiration, and water use efficiency decreased dramatically when the soybeans were exposed to salt stress for a long time. The  $g_s$  is a measure of the degree of stomatal opening and can be used as an indicator of plant water status because transpiration is sensitive to plant water potential. Generally, the large reduction of  $g_s$  is considered a major way to decrease water loss from the leaves and could be considered an adaptive character to salt tolerance (Hasanuzzaman *et al.*, 2018; Hasanuzzaman *et al.*, 2023). However, the drawback of this response is the attenuation of intracellular CO<sub>2</sub> supply for carbon fixation. In the present study, we found that the decreases of A value in the salt-exposed WT plants were higher than those in the *GmCKX13*-transgenic plants (Figure 1A). However, there was no difference in stomatal conductance (Figure 1B) or intracellular CO<sub>2</sub> concentration (data not shown) between the two genotypes under the same treatment. These results indicated that one of primary reasons for the decreased photosynthesis in soybean was stomatal closure, which prevented the CO<sub>2</sub> diffusion from the atmosphere onto the mesophyll cells. However, stomatal conductance was not an advantage for the transgenic plants to get better assimilation. It has been previously indicated that non-stomatal factors such as CO<sub>2</sub> utilization capacity also affect the final rate of carbon assimilation (Feng *et al.*, 2014).

Chlorophyll content is one of the important factors in determining plant's overall photosynthetic performance (Shao *et al.*, 2014; Zhou *et al.*, 2017) and decrease in chlorophyll contents of leaves in response to salt stress is a general phenomenon (Hameed *et al.*, 2021). Investigating this photosynthetic parameter revealed that the transgenic plants could maintain a higher chlorophyll content under high salt conditions compared to their WT counterparts (Figure 2B). This result suggests a possibly lower chlorophyll degradation rate following the stress challenge in the former group.

In addition, a better health status of the PSs in the transgenic plants, as indicated by the higher  $F_v/F_m$  value (Figure 2A) coupled with data for photochemistry of PSII and electron transport (Figures 3A, B, C), was observed. Chlorophyll fluorescence is a rapid and non-intrusive tool used to screen varieties for salinity tolerance (Baker, Rosenqvist, 2004; Tsai *et al.*, 2019). It is well known that a sustained decrease in  $F_v/F_m$  indicates the occurrence of photoinhibitory damage, in response to many environmental stresses including salinity (Tsai *et al.*, 2019; Swoczyna *et al.*, 2022). The higher  $F_v/F_m$  values imply a higher integrity of photosynthetic apparatus (Swoczyna *et al.*, 2022). Significant decreases in the  $F_v/F_m$  values at the high salt levels could be arisen from the damage of the oxygen evolving complex or in PSII reaction centers (Allakhverdiev *et al.*, 2000).

As the fluorescence behavior of any photosynthetic system could change continuously (Oukarroum *et al.*, 2012), we also looked at the  $PI_{ABS}$  values, which are considered more sensitive to environmental changes than the  $F_v/F_m$  values, thus highly correlated with the plant's health status (Masarmi *et al.*, 2022). It is assumed that species with high  $F_v/F_m$  and  $PI_{ABS}$  are species that are more adaptable to the environment than other species in the same landscape or ecosystem (Hermans *et al.*, 2003). Furthermore,  $PI_{ABS}$  incorporated with other parameters such as  $\phi_{Po}$ ,  $\phi_{Eo}$ , and  $\psi_{Eo}$  was particularly useful in revealing photosynthetic

activity behavior. Our research showed that the  $PI_{ABS}$  values of transgenic plants only fluctuated under different salt treatments. Also, the  $PI_{ABS}$  values of transgenic soybeans were significantly higher than that of their WT counterparts, particularly with high salt treatments (150 - 200 mM NaCl) (Figure 3). These indicated that the transgenic could probably sustain better photochemical efficiency of PSII and electron transport chain under the stress conditions. Values of  $\phi_{Po}$ ,  $\phi_{Eo}$ , and  $\psi_{Eo}$  that were related to the flux ratio of electron transfer were also higher in transgenic plants and in agreement with the higher  $PI_{ABS}$  (Figure 3).

Collectively, these are lines of evidence supporting the more effective light-dependent activities in photosynthesis of the transgenic plants. Therefore, the results indicated that the transformant might suffer less damage in their photosynthetic machinery and less protein degradation issues triggered by the challenged stress.

## CONCLUSION

The data in this study have revealed that the soybean *GmCKX13* activity could contribute to the better maintenance of photosynthetic performance under the salinity conditions by minimizing the reduction in chlorophyll content and stabilizing PSII photochemistry as well as the flux ratio of electron transfer activities, thus conferring the better maintenance of carbon assimilation. Therefore, the underlying mechanisms of how photosynthetic machinery gets better functional remains to be elucidated in the future work.

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