DROUGHT-RELATED PARAMETERS REVEAL BETTER DROUGHT TOLERANCE OF TRANSGENIC PLANTS OVEREXPRESSING A SOYBEAN *CYTOKININ DEHYDROGENASE* GENE

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SUMMARY

Drought stress is one of the most detrimental abiotic stresses that has undeniably negative impacts on the growth and development of soybean plants. According to previous studies, cytokinin-related genes have been proposed to play vital roles in plant response and adaptation to drought stress. From a report, the soybean gene *GmCKX13* encoding cytokinin oxidases/dehydrogenase was shown to be upregulated under drought condition. In this study, *GmCKX13*-overexpressing soybeans were used to evaluate the involvement of *GmCKX13* in plant adaptation to water scarcity condition. According to the results, the introduction of *GmCKX13* transgene conferred the transgenic plants enhanced drought tolerance capacity, which was featured with higher drought-tolerant index and better water retaining in the tissue under water deficit condition in comparison with the wild-type plants. Additionally, the transgenic soybean plants also showed lower level of intracellular hydrogen peroxide content, which was in agreement with the stronger activities of hydrogen peroxide-scavenging enzymes - catalase and peroxidase. These results indicate a promising application of *GmCKX13* in enhancing antioxidant defense system against oxidative damage caused by drought stress in plants. Taken together, therefore, it is suggested that *GmCKX13* may contribute to soybean adaptability to drought stress, which would result in crop yield improvement.

Keywords: Antioxidant enzymes, *cytokinin dehydrogenase*, drought tolerance, homologous expression soybean system, water deficit

INTRODUCTION

Soybean (*Glycine max* L. Merrill) is not only the leading commercial oilseed species among crop plants but also the largest plant source of vegetable oil and proteins for human consumption and animal feed (Vogel *et al.*, 2021). However, the quality and productivity of

soybean are affected by adverse environmental conditions. Particularly, drought stress is considered as the major constraint that decreases plant productivity. As soybean is a susceptible crop to water availability, drought can affect many physiological and biochemical processes in all stages of plant growth, which could result in the reduction of nearly 50% of its yield (Poudel *et al.*, 2023).

It has been known that under drought condition, the plants trigger certain mechanisms for morphological and physiological modification to maintain their survival (Basu *et al.*, 2016). Under such incident, plants tend to generate more endogenous reactive oxygen species (ROS), including the radical ROS-types such as superoxide and the non-radical ROS-types such as hydrogen peroxide (H_2O_2). At accumulated levels, these ROS could cause oxidative damage to plant cells by attacking proteins, lipids, carbohydrates, and DNA, thus disrupt their functions (Sharma *et al.*, 2012; Sachdev *et al.*, 2021). In response to drought stress, plants generally trigger different series of biochemical reactions to cope with the water deficiency. One common strategy that the plants use to survive through the stress period is to enhance the defensive system to reduce the excessive levels of ROS (Laxa *et al.*, 2019). Major antioxidant enzymes that are engaged include superoxide dismutase (SOD) to detoxify the superoxide, and peroxidase (POD) and catalase (CAT) to remove hydrogen peroxide (Hasanuzzaman *et al.*, 2020; Sachdev *et al.*, 2021). Therefore, measuring the activities of these enzymes are commonly carried out in evaluation of drought tolerance capacity of the plants.

Research on regulatory functions of cytokinin (CK) reveal that it acts as a negative regulator in plant root growth yet positive regulator in plant shoot growth (Ramireddy *et al.*, 2018; Liu *et al.*, 2020). Together with the key modulator, abscisic acid, CK is the additional phytohormone involved in the regulation of plant responses to drought stress. Also, in recent years, lines of evidence have been reported that CK concentration plays essential role in environmental stress-related adaptation in plants, such as water deficit stress (Hai *et al.*, 2020). The level of CK is controlled by two main enzyme lineages - isopentenyl transferases (IPTs) catalyzing the biosynthesis of CK, and CK oxidases/dehydrogenases (CKXs) being responsible for the irreversible degradation of CK (Dung *et al.*, 2012). Many studies have supported that irreversible CK degradation has a significant regulatory role (Sakakibara, 2006; Werner *et al.*, 2006). Previously, overexpression of *CKX* genes led to the drop of CK levels and resulted in the formation of longer and highly branching root systems, enhancing the accumulation of certain nutrients, and consequently increasing the tolerance to drought stress (Ramireddy *et al.*, 2018; Hai *et al.*, 2020). Among the *GmCKX* genes detected in soybean, *GmCKX13* was one of the highly upregulated genes in vegetative tissues under drought stress condition (Dung *et al.*, 2012). These findings suggest that *GmCKX13* was greatly involved in soybean response to drought and its potential modulation to improve the plant drought tolerance by genetic engineering approach remains to be explored.

In this study, the main purpose was to examine the role of *GmCKX13* in mediating soybean tolerance to drought *in planta*. To do this, the transgenic soybean plants overexpressing *GmCKX13* was used and compared with the wild-type (WT) plants regarding drought-tolerant index (DTI), the growth of shoot and roots, the relative water content (RWC) in the shoot tissue, the intracellular H_2O_2 content, and H_2O_2-scavenging enzyme activities under normal and water deficit conditions.

MATERIALS AND METHODS

Materials

The WT soybean (Williams 82) was supplied by RIKEN Center (Yokohama, Japan). The transgenic soybean seeds were generated by *Agrobacterium*-mediated transformation method

using service at Iowa State University (Ames, USA), also based on the Williams 82 background. The transgene was driven by promoter *PYK10*, which is a root-specific promoter (Nitz *et al*., 2001). Cassette P_{PYK10}-*GmCKX13-NOS* was cloned into pENTR Direction TOPO before it was mobilized to pTF101.1gw1 vector for transformation.

Plant growth

The seeds were soaked in water at 37 °C for 30 min, then being sown to Tribat soil contained in plastic trays for germination. After germination, all plants were cultivated under greenhouse condition (temperature range 28-33 °C, relative humidity range 60-70 %, natural photoperiod). The seedlings were watered once per day with the same amount of water until they were at right stage for experiments. The soil moisture content (SMC) was measured by using a soil moisture meter (Thu *et al*., 2014).

Drought-tolerant index determination

The drought-tolerant index (DTI) was determined based on the method described in previous study (Thu *et al*., 2014) with some modifications. In brief, the Gm*CKX13*-overexpressing and WT soybean plants were grown separately in plastic tubes, which were 25 cm in height and 30 cm in diameter (*n* = 30 plants per genotype). When the plants were 12 days old, drought stress was applied by stopping irrigation for the next 27 days, followed by 27 days of re-irrigation. During the drought duration, percentage of non-withered plants after every two days were recorded, starting from the first day of treatment. Similarly, during the re-irrigation period, percentage of recovered plants were also recorded on the alternate day. DTI values were calculated using the formula specified in Thu *et al*. (2014).

Shoot/root growth and shoot relative water content determination

For this assay, 20 plants of each genotype were grown in plastic tubes (80 cm in height and 10 cm in diameter, one plant per tube) using Tribat soil. Both transgenic and non-transgenic

plants were grown under well-watered condition for 12 days (Thu *et al*., 2014). After that, the plants were divided into two equal sets. One set was used as control group which remained to be watered normally (i.e. the SMC was maintained within the range of 60-70%). The other set was exposed to drought stress by withholding water in the next 15 days.

After the drought treatment, the plants were gently removed out of the soil for recording fresh weights (FWs) of the shoots as well as the length of the tap root and shoot tissues. Then, the shoots were soaked in deionized water for 24 h at room temperature before their turgid weights (TWs) were obtained. After that, the samples were dried in oven for 48 h at 65 °C to record their dry weights (DWs) (Thu *et al*., 2014). The tissue RWC was calculated using the following formula:

$$RWC\ (\%) = \frac{FW - DW}{TW - DW} \times 100$$

Cellular hydrogen peroxide content determination

The H_2O_2 content was determined based on the method described by Patterson and others (1984). The extraction of cellular H_2O_2 was carried out by homogenizing 0.2 g of soybean leaves (0 day and 15 days since drought application) in 2 mL PBS solution (0.1 M, pH 7.4) using pre-chilled mortar and pestle. The cellular H_2O_2 solution was collected from the supernatant of crude extract solution following centrifugation. The reaction solution was established containing cellular H_2O_2 solution and 0.1 % Titanium Sulfate in 20% H_2SO_4 (v/v). After centrifugation, the absorbance of the assay was measured at 410 nm. Concentration of endogenous H_2O_2 was inferred from the standard curve of H_2O_2 solution. The number biological replications were four for each group of samples (*n* = 4).

POD and CAT activity determination

The antioxidants enzymatic activities were determined from a crude enzyme solution extracted by homogenizing 0.2 g of soybean

leaves in 2 mL cold extraction buffer (pH 7.0, containing 1 mM EDTA and 2% Polyvinylpyrrolidone (PVP-8000) in 50 mM Potassium phosphate buffer), using pre-chilled mortar and pestle, followed by centrifugation for obtaining sample supernatant.

POD activity was determined following the method described in Shannon and colleagues (1966) and calculated using the formula of Rodríguez *et al*. (2001). The reaction mixture consisted of POD substrate solution (which included acetate 0.1 M, *o*-dianisidine 0.5% and fresh H_2O_2 0.1 M), and 100 µl of the sample supernatant solution was prepared and incubated at 37 °C for 30 min. Peroxidase activity was measured based on the change in absorbance at 460 nm over 3 min. Four biological replicates per treatment were used for this assay.

The method for analysis of CAT activity was described in study of Wang and others (2012). For this assay, reaction solution consisted of 2.9 mL CAT substrate solution (potassium phosphate buffer 1 M, EDTA 0.1mM, fresh H_2O_2 0.1 M) and the sample supernatant. Catalase activity was monitored following the decreased absorbance of H_2O_2 (extinction coefficient 0.0394 mM^{-1} cm^{-1}) in each reaction at wavelength of 240 nm over 1-minute duration. The number biological replications were four for each group of treatment ($n = 4$).

For total soluble protein quantification in order to calculate enzyme activities, Bradford method was used in accordance with previous research (Xuan *et al*., 2019).

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.

RESULTS AND DISCUSSION

The transgenic plants displayed higher DTI value

To generally evaluate the drought-tolerant degree of the transgenic plants compared to WT,

DTI assay was carried out. This method has been deployed at least in soybean and rice studies (Binh, Muoi, 1998; Mau *et al*., 2010; Thu *et al*., 2014), and the DTI values could be considered as an indicator for depicting the potential survival and recovery rates of plants when being exposed to drought stress. Higher DTI value would indicate a possible better tolerance capacity of the genotype against water deficiency (Thu *et al*., 2014).

In the DTI assay, both WT and transgenic plants were grown under well-watered condition for the first 12 days, followed by a 27-day period of drought stress by non-irrigation and a 27-day period of re-irrigation. To ensure the experiment had been set up properly, the SMC was monitored. As shown in Figure 1A, the SMC was maintained around 70% during the normal period and dropped to approximately 25 % at the end of the drought application. It has been noted that at this stage, more than 50 % of the studied WT plants became wilted whereas a lower proportion of the transgenic plants showed the apparent drought symptoms. In addition, the latter also showed a higher number of plants recovered during the re-irrigation period. The analyses revealed that DTI value of transgenic line (0.3699) was nearly 3-fold higher compared to the corresponding value of the WT (0.1349) (Figure 1B). This finding indicated better potential tolerance of the transgenic plants under limited water supply conditions.

The transgenic plants acquired longer root growth and better water reservation in the tissue under drought conditions

As reported from former studies, shoot length and leaf area of plants is usually reduced under drought stress and the reduction could be up to 25 % (Specht *et al*., 2001; Wu *et al*., 2008; Anjum *et al*., 2017; Bhusal *et al*., 2020;). However, under this unfavorable condition, promotion of the root growth could be observed (Jaleel *et al*., 2007; Zhan *et al*., 2015; Yang *et al*., 2018). Such responses are suggested to provide the plant benefits under the stress condition by increasing the chance to extract

water from deeper soil layers and compensate for the water loss from transpiration, as well as save the energy for basic metabolism and plant survival (Shao *et al*., 2008; Yang *et al*., 2021).

As the shoot and root characters could reflect the potential capacity of plants to cope with stress, therefore, shoot and root assay was also conducted in this study.
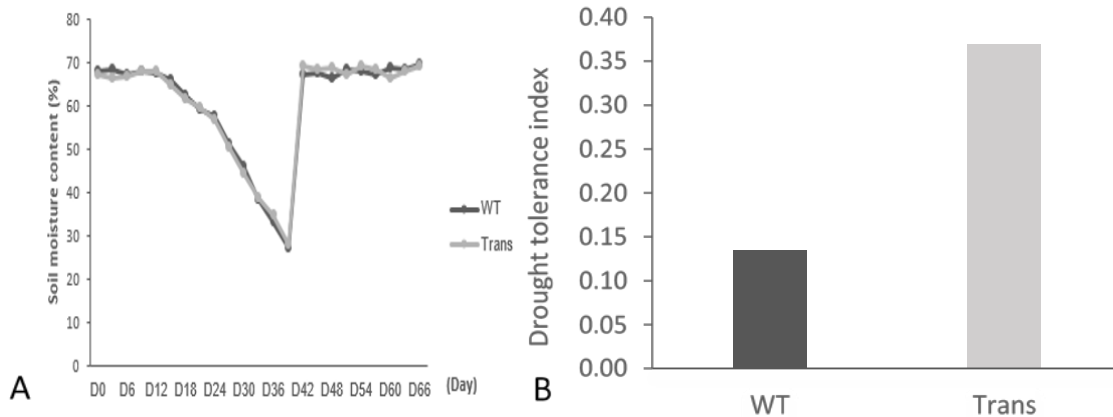

Figure 1. Drought-tolerant index of *GmCKX13*-transgenic (Trans) and wild-type (WT) soybean plants ($n = 30$). The 12-day-old plants were exposed to a 27-day drought stress, followed by 27-day re-irrigation. (A) Soil moisture content measured every 3 days during the experiment. (B) DTI values of WT and transgenic plants.
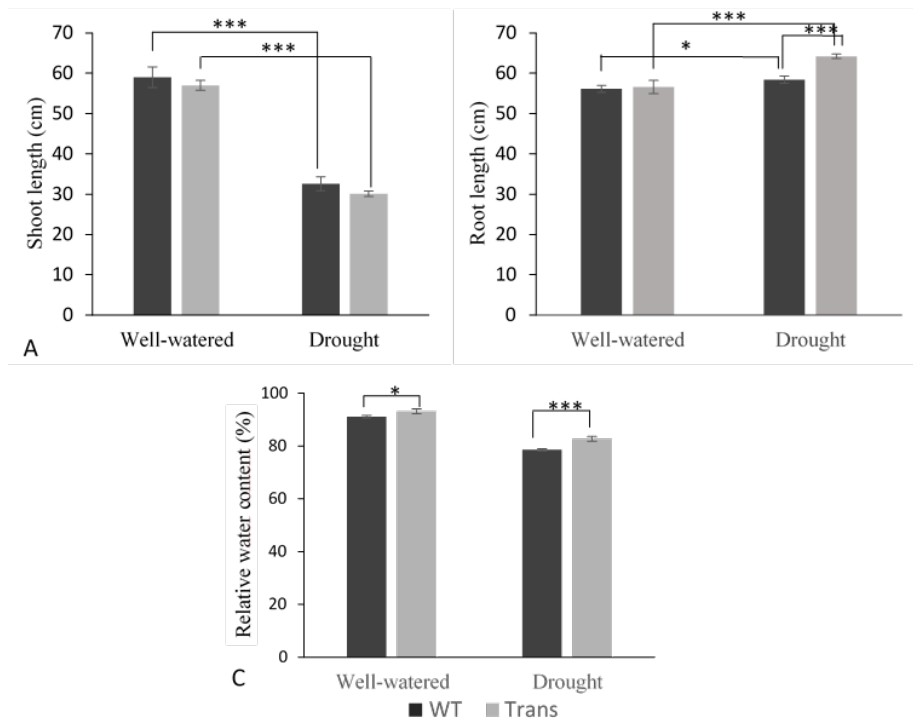

Figure 2. Shoot- and root- related characters of the *GmCKX13*-transgenic (Trans) and wild-type (WT) plants under normal and drought stress conditions. (A) Average shoot lengths. (B) Average root lengths. (C) Relative water contents in the shoot tissue. The asterisk indicated significant difference (*: p-value < 0.05; ***: p-value < 0.001). The values were mean ± SE of twenty biological replicates. Twelve-day old seedlings were applied drought stress for 15 days.

According to the data, the drought application resulted in significant reduction in shoot length (by *ca*. 26 cm in both studied genotypes) and yet increase in primary root length (by *ca*. 2.3 cm in the WT and by *ca*. 7.7 cm in the transgenic plants) compared to their counterparts that were grown under normal conditions (Figures 2A, 2B). In addition, it was noted that there was no difference in either shoot or root lengths between two studied genotypes under the same treatment, apart from a substantially longer average taproot in the transgenic plants than the corresponding value of the non-transgenic plants upon the water stress exposure (Figure 2A, 2B). It is noted that the employed *PYK10* promoter to drive the expression of the transgene is a root-tissue specific promoter (Nitz *et al*., 2001). It may explain, therefore, why the transgenic plants exhibited no major alterations in shoot-related parameters over the WT plants.

The drought tolerance responses of the *GmCKX13*-transgenic versus the WT plants were also evaluated and compared by examining the RWC in the aerial tissues, another common drought-related parameter. As shown in Figure 2C, the RWC values of the transgenic plants were considerably higher those of the WT plants not only under the normal (by 2.04 %) but also the drought conditions (by 4.18 %). These obtained results suggested that under water deficit condition, the transgenic plants had better capacity in retaining water.

The transgenic plants had lower endogenous hydrogen peroxide contents and stronger POD and CAT activities

To assess the drought-induced oxidative stress degrees, we analyzed endogenous H_2O_2 contents in the leaf tissue. H_2O_2 is the common ROS that is excessively produced under the stress conditions (Xuan *et al*., 2019). The results showed that the drought-treated WT plants suffered significantly higher levels of H_2O_2 than the corresponding concentration measured in the stressed *GmCKX13*-transgenic plants although the average H_2O_2 levels of these studied genotypes were similar under the normal

conditions (0-day drought stress) (Figure 3A). This result implies that the transgenic plants might suffer a lower level of cellular damage than the WT plants under the drought stress, at least due to H_2O_2 attack.

In plants, POD and CAT are the enzymes responsible for cellular H_2O_2 removal. Therefore, we further evaluate activities of these workhorses. Figure 3B showed that there was no significant difference in POD activities of the WT versus the transgenic soybean samples under normal growth condition (0-day drought stress). After 15-day-drought treatment, the activities of PODs were enhanced by 1.7-fold and 2.6-fold in the WT and transgenic plants, respectively. Therefore, under the stress condition, the latter displayed significantly higher POD activities. A similar observation was also observed for the CAT activities, with a higher degree of increased enzyme activity in the transgenic group (Figure 3C). Taken these data together, the lower rate of H_2O_2 accumulation in the stressed transgenic plants at least thanks to the stronger ROS removal activities of POD and CAT enzymes. The finding also evidenced for a better tolerance to drought and probably an enhanced antioxidant capacity in the transgenic plants to defend the oxidative damages caused by water deficit condition.

Our result was also similar to previous study conducted by Eltayeb and others (2011) on drought-tolerant potato overexpressing *Arabidopsis* cytosolic gene, which also reported a lower cellular H_2O_2 content in the transgenic plants, compared with the WT potatoes. In another study, it was also provided for the evidence of the transgenic *Arabidopsis* carrying *35S::GmNAC019* with better post-drought survival rate accompanied with remarkably higher activities of CAT and POD and lower H_2O_2 content under drought stress condition (Xuan *et al*., 2019). Therefore, it is suggested the considerable enhancement of antioxidant activities in the transgenic soybeans harboring *GmCKX13* gene during water deficit condition would contribute to the better tolerance of the plants.
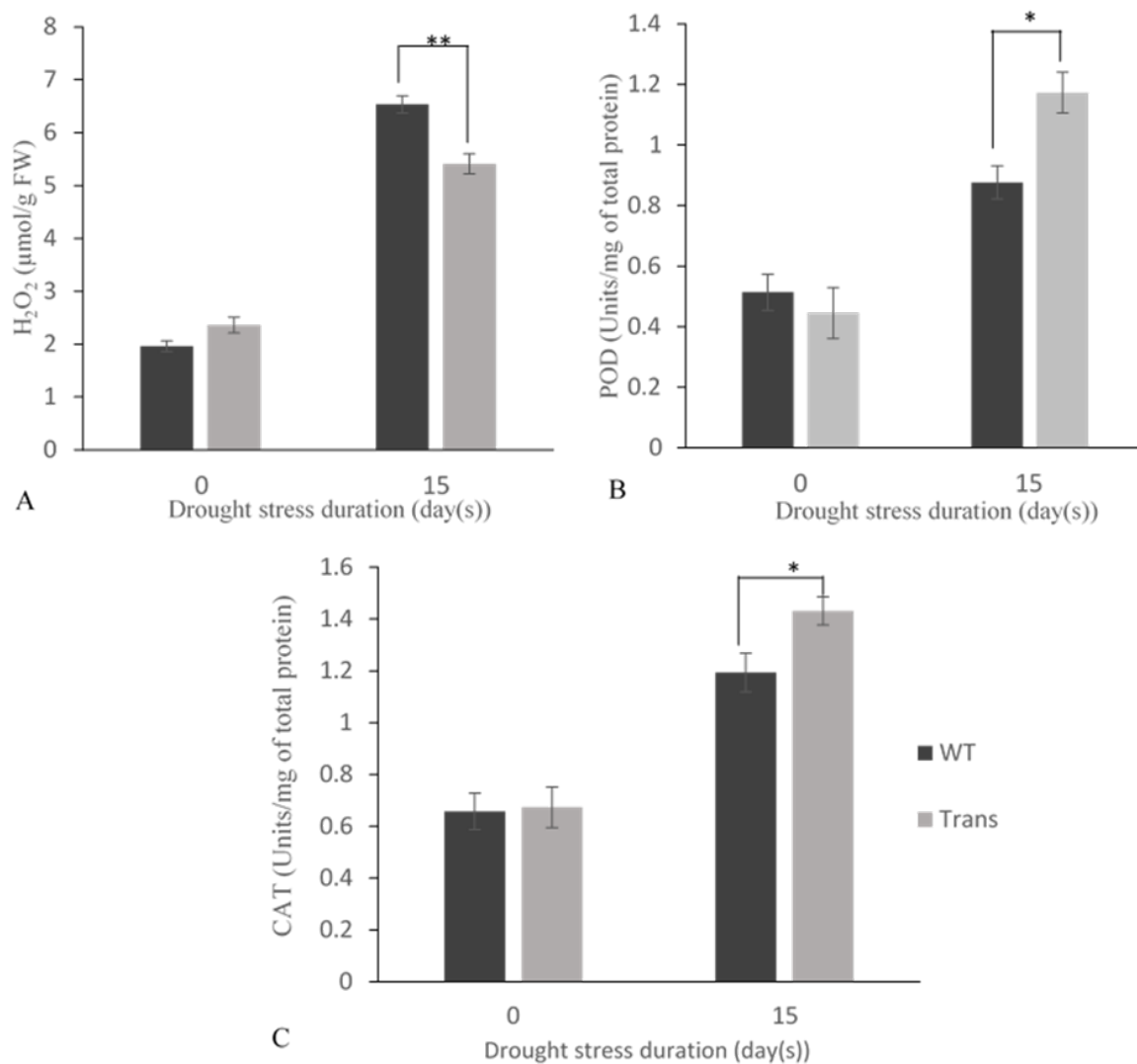
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Figure 3. Cellular hydrogen peroxide (H_2O_2) contents and activities of H_2O_2-scavenging enzymes in the *GmCKX13*-transgenic (Trans) and wild-type (WT) plants under normal and drought stress conditions. (A) H_2O_2 contents in the leaf tissue. (B) Peroxidase (POD) activities. (C) Catalase (CAT) activities. The asterisk above the column indicates that the value was significantly different (*: *p*-value < 0.05; **: *p*-value < 0.01). The values were mean ± SE of four biological replicates.

CONCLUSION

In summary, the findings indicate that *GmCKX13*-transgenic soybean plants might acquire better drought tolerance than the WT plants, which are supported by the better capacity of remaining water in the tissue, lower endogenous H_2O_2 content coupled with stronger H_2O_2-scavening activities by catalase and peroxidase enzymes under the water scarcity conditions. The DTI values were also in agreement with these analyses, whereby the transgenic plants had much higher tolerance index. These results, therefore, indicate the potential application of *GmCKX13* expression modulation to enhance drought tolerance capacity in crop plants. To gain more insightful understanding of the mechanism of CKX13-mediated regulation against the drought stress, further studies should be conducted with the

focuses on investigation of stomatal characters, biochemical changes, productivity and gene expression activities.

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