Ectopic expression of *AtCIPK23* enhances drought tolerance via accumulating less H_2O_2 in transgenic tobacco plants

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ABSTRACT. Drought is a major threat to agricultural crop growth and productivity. However, the molecular mechanism behind such effects remains largely unknown. Plant CBL (Calcineurin B-Like)-interacting protein kinases (CIPKs) are believed to play an important role in plant drought tolerance and signalling transduction. Here, we report the influence of a member of the CIPK family, *AtCIPK23*, in drought stress responses in tobacco. Transgenic tobacco plants over-expressing *AtCIPK23* showed enhanced tolerance to drought stress compared with wild-type plants. After drought stress treatment, the survival rates and levels of chlorophyll, proline, and soluble sugar in three transgenic lines were significantly higher than those in WT plants. Additionally, in the transgenic plant lines the accumulation of H_2O_2 was lower, and the expression levels of NtSOD, NtCAT and NtAPX were higher compared with wild-type plants under drought stress. A quantitative RT-PCR analysis revealed that over-expression of *AtCIPK23* in tobacco induced the expression of the NtDREB, NtLEA5 and NtCDPK2 genes. Therefore, *AtCIPK23* perhaps is involved in the plant response to drought stress via regulating the expression of stress-related genes.

Key words: *AtCIPK23*, Drought, Tobacco (*Nicotiana tabacum* L), transformation, ROS

INTRODUCTION

In agricultural practice, crops often encounter various environmental stresses, such as unfavourable temperatures, high salinity, and drought. These stresses negatively affect the growth and development of crops, leading to decreased crop yield. To increase the survival rates and improve yield, agricultural plants have developed various mechanisms to adapt to these abiotic stresses, which involve various signalling transduction cascades and the activation of stress-responsive molecular networks (Zhu, 2002; Albrecht et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007; Vij and Tyagi, 2007; Xiang et al., 2007).

Calcium signaling play important roles in plant physiological and developmental processes. As a secondary messenger, the increased cytosolic free Ca^{2+} concentration can transduce the extracellular stimuli and regulates various molecular network responses (Sanders et al., 2002; Albrecht et al., 2003; Berridge et al., 2003; Kim et al., 2003a, 2003b; Cheng et al., 2004; Kolukisaoglu et al., 2004; Tuteja and Mahajan, 2007; Kudla et al., 2010). In higher plants, there are several main Ca^{2+} sensors families identified, including calmodulins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and calcium-dependent protein kinases (CDPKs) (Magnan et al., 2008; DeFalco et al., 2010; Conde et al., 2011; Boudsocq and sheen, 2013; Zhang et al., 2014).

To mediate Ca^{2+} signaling functions, the CBL proteins can specifically target CBL-interacting protein kinases (CIPKs, also known as PKS, protein kinase) and activate its activity to transduce calcium signals by autophophorylating or phosphorylating downstream components in response to various stimuli (Kim et al., 2000; Albrecht et al., 2003; Batistic and Kudla, 2004; Pandey et al., 2004; D'Angelo et al., 2006; Cheong et al., 2007; Batistic et al., 2008; Luan, 2009; Deng et al., 2013; Zhang et al., 2014).

In higher plants, 43 CIPKs in maize (Chen et al., 2011), 34 CIPKs in rice (Kanwar et al., 2014; Zhang et al., 2014), 27 CIPKs in poplar (Yu et al., 2007), and 26 CIPKs in Arabidopsis (Kolukisaoglu et al., 2004) were identified. Plant CIPKs play crucial roles in plant ion homeostasis and transportation across the plasma membrane or tonoplast to increase plants' tolerance to various abiotic stress (Zhang et al., 2014). AtCIPK24 (AtSOS2) and its counterparts in higher plants are proved to increase salt resistance. Interacting with AtCBL4 (AtSOS3), AtCIPK24 (AtSOS2) can activate the plasma membrane-localized Na+/H+ antiporter AtNHX7(SOS1) and vacuolar H+-ATPase resulting in enhanced salt tolerance in Arabidopsis (Qiu et al., 2002; Batelli et al., 2007; Kudla et al., 2010). Similarly, the formation of AtCBL10 and AtCIPK24 (AtSOS2) complex also improves Arabidopsis shoots tolerance to salt stress (Kim et al., 2007; Quan et al., 2007). The homologous genes of AtCIPK24 (AtSOS2), such as MdCIPK6L, MdSOS2, and ZmCIPK16 (Zhao et al., 2009; Hu et al., 2011; Wang et al., 2012) have been reported similar functions as it in increasing plants' tolerance to salt stress. Additionally, plant CIPKs have also been proved to function in cellular K+ homeostasis. In Arabidopsis, the CBL1/CBL9-CIPK23 complex can interact with and activate K+ transporter protein AKT1 by phosphorylation on the membrane to promote K+ transport into the plant cell (Li et al., 2006; Xu et al., 2006). In rice, the OsCBL1/OsCIPK23 complex can activate K+ channel OsAKT1 and enhance OsAKT1-mediated K+ uptake (Li et al., 2014). Similar results have also been found in HbCIPK2 and SlSOS2 in cell K+ homeostasis (Huertas et al., 2012; Li et al., 2012). Moreover, *AtCIPK23* can also phosphorylate T101 of CHL1 to maintain a low-level primary response in response to low nitrate concentrations (Yu et al., 2014).

Several reported plant CIPK family genes could be activated by other abiotic stresses and plant hormone, including auxin and abscisic acid (ABA) (Luan et al., 2009; Weinl and Kudla, 2009). In rice, OsCK1/OsCIPK3 is involved in responses to diverse signals including cold, light; cytokinin's, sugars and salts (Kim et al., 2003b). Down-regulated expression of OsCIPK23 conferred a drought stress sensitive phenotype, and over-expression resulted in up-regulated expression of several drought tolerances related genes (Yang et al., 2008). In cassava, most MeCIPKs were induced by drought stress (Hu et al., 2015), underlying their roles in response to this stimuli. Arabidopsis CIPK6 is critical for auxin transportation, influencing root morphogenesis and salt stress response (Tripathi et al., 2009; Chen et al., 2013). AtCIPK26 has been shown involved in ABA signaling during seed germination by interacting with ABI1, ABI2, and ABI5 (Lyzenga et al., 2013). In conclusion, all these evidences have proved that plant CIPKs are vital for plant response to various stimuli. However, the function of *AtCIPK23* in response to drought stress remains still unknown. In this study, we report that *AtCIPK23* could be

involved in transgenic tobacco plants' response to drought stress, and our results suggest that *AtCIPK23* could be a multistress induced gene that is critical for plant abiotic stress adaption.

MATERIALS AND METHODS

Construction of *AtCIPK23* overexpression vectors and tobacco genetic transformation

For *AtCIPK23* overexpression vectors construction, full-length coding sequence of the *AtCIPK23* gene was cloned using RT-PCR. The resulting amplicons was then inserted into pCAMBIA 1300 plant expression vector under the control of Super promoter (Chen et al., 2009). This vector was introduced into Agrobacterium tumefaciens strain EHA105, and transgenic tobacco plants were generated by Agrobacterium-mediated transformation as described in previous study (Bao et al., 2017).

Transgenic tobacco plants were monitored on MS medium containing 50 mg/L hygromycin, and the survival plants were then verified by PCR. T1 transgenic tobacco seeds were surface-sterilized and germinated on MS medium supplemented with 50 mg/L hygromycin, and transgenic lines with a 3:1 (resistant: sensitive) segregation ratio were selected to produce seeds. The T3 transgenic lines which exhibited 100% hygromycin resistance were then chosen for further study.

Plant material and stress treatment

The tobacco wild-type K326 and the *AtCIPK23*-over-expressing transgenic tobacco lines KA13, KA14 and KA44 were obtained as described above, and the expression levels of *AtCIPK23* in the transgenic lines and the wild-type were monitored by qRT-PCR using gene-specific primers (Supplementary data).

Tobacco seeds from various transgenic lines and wild-type were surface-sterilized and planted in 20 cm diameter plastic pots filled with 3 kg of nutrient soil. For the drought treatment, water was withheld from the tobacco seedlings for 12 days after 40 days of normal water supply. Then, the tobacco seedlings were re-watered, and the survival rate was calculated after 3 days of rewatering.

On the seventh day after the drought treatment, leaf samples from 10 plants of each transgenic line and wild-type were collected. Total chlorophyll was determined by SPAD Chlorophyll Meter (SPAD502 Plus, Konica Minolta, Japan). The content of proline was measured as described by Claussen (2005). 0.2 g leaf samples (fresh weight) from treatments and control were collected, ground in a mortar with quartz sand and 5 ml 3% (w/v) aqueous sulfosalicylic acid solution, and transferred to test tubes. The closed test tubes were kept in a boiling water bath for 10 min, cooled to room temperature (21°C) and filtered. The clear filtrate was then transferred to another clear test tubes and constant-volume to 5 ml with 3% (w/v) aqueous sulfosalicylic acid solution. The proline concentration was determined immediately at a wavelength of 546 nm with a LG-721 spectrophotometer. The amount of soluble sugar was measured as described by Yang et al. (2007). Tobacco seedling leaves from treatments and control were collected, and treated at 105°C for 20 min and further dried at 80°C for 8 h. Then, 0.1 dry materials from each sample was boiled in 40 ml ddH_2O for 40 min, and filtered. The filtrates were then transferred to 50 ml volumetric flasks and constant-volume to 50 ml with ddH2O. Total amounts of soluble sugar were measured at 620 nm on a LG-721 spectrophotometer.

RNA extraction and qRT-PCR analysis

Total RNA was isolated from leaves of 30-day-old tobacco seedlings and from seedlings after 7 days of the drought treatment by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA generation and real-time PCR were performed according to Lu et al. (2015). In brief, cDNA was generated by using TaqMan Reverse Transcription Regents kit (Applied Biosystems, Foster City, USA: ABI), and 5 ng of the cDNA template was applied for quantitative assays using the SYBR Green Master Mix (ABI, PN4309155) with an ABI 7900 sequence detection system according to the manufacturer's instructions(ABI). The tobacco Actin gene was used as an internal control, and the relative expression of the tested genes between replicates examined were evaluated according to the relative quantification method (Delta-Delta CT, Livak and Schmittgen, 2001).

Gene-specific primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/input.htm) as described in Supplementary data.

Water loss assay

Leaves from various transgenic lines and wild-type of 30-day-old seedlings were collected and left to dry for 12 h at room temperature. The water loss rates were then calculated according to following formula: water loss rate= (total weight - fresh weight)/total weight × 100%.

Leaf relative electrical leakage assay

Tobacco leaf samples from wild-type and the transgenic lines were collected after 4 days of the drought treatment. Ten leaf discs 10 mm in diameter were obtained from each sample, and the electrical conductivity (R1) was measured using the electrical conductivity metre (Sino measure Product, Hangzhou, China) after soaking in ddH2O for 12 h. Then, the discs were incubated in boiling water for 30 min, and the electrical conductivity (R2) was determined. The relative leaf electrical leakage=R1/R2 × 100%.

Measurements of H_2O_2 Production

H_2O_2 was detected by DAB staining as described previously (Guan and Scandalios, 2000; Zou et al., 2015). Tobacco seedling leaves from drought treatments and control were incubated in DAB solution (1 mg/mL 21, pH 3.8; Sigma-Aldrich) for 8 h in dark at 28°C, and then dipped into boiling 80% (v/v) ethanol for 10 min. The leaves were extracted with 80% (v/v) ethanol after cooling, and photographed.

RESULTS

Transgenic lines overexpressing *AtCIPK23* demonstrated enhanced drought tolerance

To monitor the water loss rate, tobacco leaves from three transgenic lines and wild-type (WT) were collected and left to dry for 12 h. The results showed that the relative water loss of all tested samples gradually increased during the testing time. The water loss rate of the WT K326 was significantly higher than that of all three transgenic lines at all-time points (Figure 1A). The average water loss rate of WT leaves was approximately 55% after 12 h, compared with 47%, 40% and 34% in the three transgenic lines, KA13, KA14 and KA44, respectively. This result indicated that the transgenic lines overexpressing *AtCIPK23* lost less water under drought stress and eventually exhibited drought tolerance.

To investigate whether *AtCIPK23* can increase drought tolerance in transgenic tobacco plants, three *AtCIPK23* transgenic lines and wild-type (WT) were treated with drought stress. There were no obvious morphological or developmental differences between the three transgenic lines and WT plants under normal growth conditions. During a 12-d period of drought treatment, WT plants showed a much more sensitive phenotype compared with the three transgenic lines. At 4-d after the drought treatment, the WT plants started to wilt, and this drought-sensitive phenotype was obvious (Figure 1B). After the drought stress treatment, all WT plants wilted, and their leaves became chlorotic, while plants from the three transgenic lines were turgid, and their leaves remained green.

After re-watering for 3 days, the plants from the transgenic lines recovered more quickly than the WT plants (Figure 1C). The survival rates of three transgenic lines KA13, KA14 and KA44 were 67%, 78%, and 89%, respectively, which were significantly higher than the recovery rate of 30% observed in WT K326.

The determination of relative electrical leakage of the tobacco leaves showed that the WT had significantly higher leakage than the transgenic lines KA14, KA 13 and KA44 (Figure 1D). Meanwhile, the chlorophyll, proline and soluble sugar contents of the transgenic plants were significantly higher than those of the WT plants at the 7th day without watering (Figures 1E, 1F and 1G).

The lower electrolytic leakage and higher concentrations of proline and soluble sugar in transgenic lines suggested that *AtCIPK23* improved the membrane integrity and maintained higher osmotic pressure in cells to enhance drought stress tolerance.

Figure 1 *AtCIPK23* gene enhanced drought tolerance in tobacco and Physiological indices determination. (A) Relative water loss rate, (B) and (C) the phenotype of tobacco after 7 days drought stress treatment and 5 days

re-watering. (D) relative electrolytic leakage. (E) and (F) the concentration of proline and soluble sugar. (G) chlorophyll content. Each data point represents mean ± SE (n=3). Asterisk indicates significant difference relative to WT.

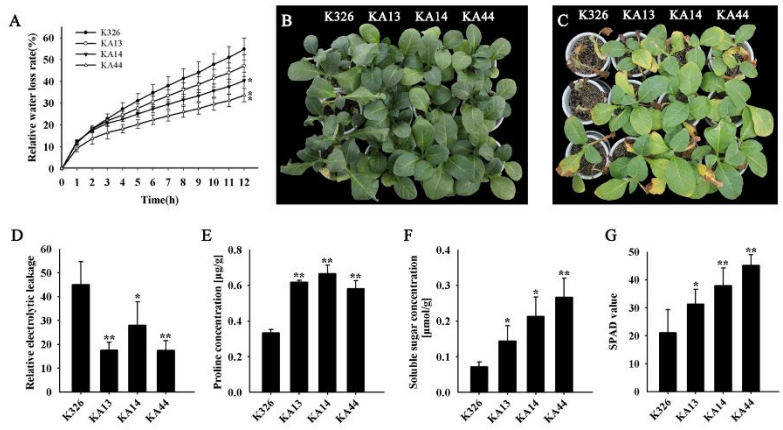

Figure 1. *AtCIPK23* gene enhanced drought tolerance in tobacco and Physiological indices determination.

Transgenic lines accumulated less H_2O_2 than WT under drought stress

The production of H_2O_2 in leaves after the drought treatment was determined using the 3,3'-diaminobenzidine (DAB) uptake method according to previous studies (Guan and Scandalios, 2000; Zou et al., 2015). As shown in Figure 2A, after the drought treatment, the transgenic tobacco plants accumulated less H_2O_2 in the tobacco leaves than the WT plants.

NtSOD, NtCAT and NtAPX encode superoxide, catalase and peroxidase, respectively, which are three key ROS scavenging enzymes that function to detoxify ROS. The expression levels of NtSOD, NtCAT and NtAPX were measured, and the results showed that the transgenic lines exhibited higher expression levels of these genes, especially NtCAT and NtAPX, under drought stress compared to WT (Figures 2B, 2C, 2D). These data suggested that the elevated expression level of ROS scavenging genes resulted in less accumulation of H_2O_2, and eventually enhanced drought tolerance in the transgenic tobacco plants.

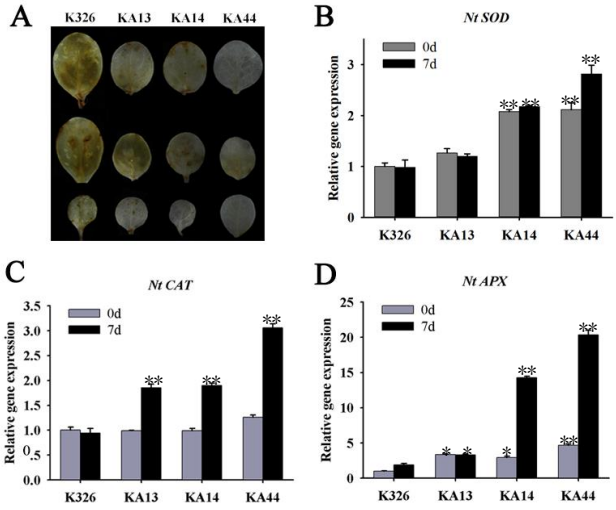

Figure 2. DAB staining and gene expression analysis of NtSOD、NtCAT and NtAPX. (A) DAB staining. (B), (C) and (D) gene expression analysis of NtSOD、NtCAT and NtAPX. Each data point represents mean ± SE (n=3). Asterisk indicates significant difference relative to WT.

Gene expression of drought related genes were increased obviously under drought stress

To further explore the reasons that the transgenic tobacco plants exhibited enhanced drought tolerance, the expression levels of three drought related genes, NtDREB, NtLEA5 and NtCDPK2, were determined using qPCR. The results showed that the expression levels of these three genes were much higher in the transgenic lines than in WT under drought stress (Figure 3). This result suggested that the expression of NtDREB, NtLEA5 and NtCDPK2 may be directly or indirectly regulated by *AtCIPK23*, leading to the enhanced drought tolerance in the transgenic tobacco plants.

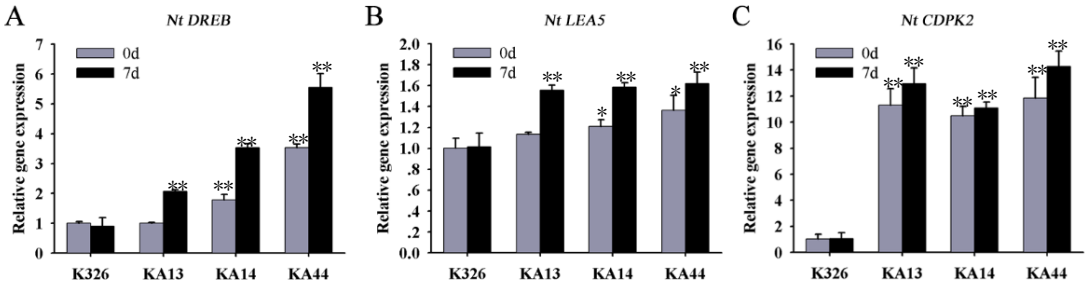

Figure 3. Expression profiles of three drought relative genes, NtDREB(A)、NtLEA5(B) and NtCDPK2(C), in WT and transgenic lines after 7 days drought stress. Each data point represents mean ± SE (n=3). Asterisk indicates significant difference relative to WT.

DISCUSSION

AtCIPK 23 responses to multiple abiotic stresses

Plant CIPKs widely participate in signal transduction under multiple stresses (Cheong et al., 2003; Kim et al., 2003a; Cheong et al., 2007; Yang et al., 2008; Cheong et al., 2010; Tsou et al., 2012; Chen et al., 2013; Tai et al., 2016). In Arabidopsis, AtCIPK 23 was identified as a positive regulator of AKT1, and plays a key role in Arabidopsis potassium uptake (Li et al., 2006; Xu et al., 2006). AtCIPK 23 overexpression in potato, sugarcane and tobacco can significantly increase the ability of transgenic plants against low potassium stress (Wang et al., 2011; Li et al., 2014; Xue et al., 2016). Apart from potassium, AtCIPK 23 also involved in the increasing high-affinity nitrogen transport capacity under nitrate deficient conditions (Ho et al. 2009). Interestingly, in Arabidopsis, when the CBL1/CBL9-CIPK23-AKT1 pathway was inhibited, the plant stomatal response was altered, and plants exhibited drought tolerance (Cheong et al., 2007). Our results also showed that tobacco plants over-expressing *AtCIPK23* exhibit greater drought tolerance than wild-type plants with higher chlorophyll content and recovery rate (Figure 1). All these results indicate that *AtCIPK23* involves in multiple abiotic stimuli. However, it remains unclear how *AtCIPK23* functions in drought stress responses.

A possible role of *AtCIPK23* played in plants drought resistance

There are three different mechanisms for plants to adopt to fight against drought stress: a) to adjust the stomata opening-closure to control water loss rate, b) to trigger an ABA-dependent signaling pathway, and c) to induce the expression of drought-response genes (Yang et al., 2008). Many stress-related genes, such as WRKY, LEA, DREB, NCED, and Rd29A, have been shown to be induced under abiotic stresses (Liu et al., 1998; Yang et al., 2008; Li et al., 2015; Kim et al., 2016). Furthermore, transgenic plants carrying the Arabidopsis DREB1A gene and the stress-inducible promoter rd29A improved drought tolerance in tobacco (Kasuga et al., 2004). A previous report showed that NtLEA5 and NtCDPK2 were up-regulated under drought stress treatment compared to wild-type (Huang et al., 2010). The results of these studies indicate that the up-regulation of these drought related genes could result in drought tolerance in plants. In our study, the expression levels of three drought relative genes NtDREB, NtLEA5 and NtCDPK2 significantly increased in transgenic tobacco plants under drought stress. In addition, the water loss assay in our study showed that the leaf relative water loss rate of transgenic plants were significantly lower than wild type K326 (Figure 1A). These results indicate that *AtCIPK23* can adjust stomata opening and enhance plant drought-resistance ability.

Osmotic substances such as proline and soluble sugar play key role in plant drought response, and accumulation of which is beneficial to scavenging reactive oxygen species, reducing cell osmotic potential and maintaining

water content of leaves under stress (Babita et al., 2010). Proline can protect plants from various stresses by functioning as an osmolyte in the cytosol. Many studies have observed that plant proline content increased under drought stress (Yoshiba et al. 1997; Man et al. 2011). In addition, the increase of soluble sugar content in plant cells can also maintain water content in leaves, keep plant cell photosynthesis rate together with higher chlorophyll content, and eventually help plants to survival under drought stress. These results suggest that *AtCIPK23* can preserve plant cell in suitable condition to carry out basic physiological processes.

As electrolyte leakage (EL) is the result of membrane damage, its' content is widely used as an indicator of plant cell membrane damage (Bao et al., 2017). Previous reports showed that PmLEAs and ZmDHN2b overexpression in tobacco leading to lower electrolyte leakage content than wild-type under drought stresses (Xing et al., 2011; Bao et al., 2017). Similarly, in our study, the EL was lower in transgenic tobacco plants than in wild type indicating that *AtCIPK23* can protect plant cell membranes from damage.

Low levels of ROS accumulation in living plant cells is important for plant growth and development (Mittler, 2002; Apel and Hirt, 2004). ROS are generated under stress, and ROS-scavenging pathways are responsible for eliminating excessive ROS during this process (Mittler et al., 2004; Miller et al., 2010). Several studies have shown that transgenic plants exhibit enhanced tolerance to salt and drought stresses, possibly as a result of decreased ROS accumulation (Asano et al., 2012; Zou et al., 2015). In this study, *AtCIPK23* transgenic tobacco plants showed lower ROS accumulation and exhibited enhanced drought stress. Meanwhile, three ROS scavenging related genes, NtSOD, NtCAT and NtAPX, were up-regulated in the transgenic plants. This result indicates that *AtCIPK23* may indeed enhance plants' drought stress tolerance via ROS-scavenging pathways (Figure 2).

Altogether, the results of our study suggest that *AtCIPK23* involves in plants' response to drought stress via a) and c) mechanism described above.

CONCLUSION

In conclusion, overexpression of *AtCIPK23* significantly enhances the tolerance of transgenic tobacco plants in response to drought stress through regulation of ROS eliminating genes and drought resistance-related gene expression. It is likely that *AtCIPK23* acts as a positive regulator in drought-resistance process. Further studies including identification of *AtCIPK23* targets will unveil the molecular basis of the role of *AtCIPK23* in plant response to drought stress.

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