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ZmSPK1, a member of plant *SnRK2* subfamily in maize enhances tolerance to salt in transgenic *Arabidopsis*

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Abstract

SnRK2s play a key role in the plant stress signaling transduction pathway. In previous study, ZmSPK1, a SnRK2 member has been cloned from maize. Expression pattern analyses showed that *ZmSPK1* is induced by mannitol, salt and ABA. To investigate its role under stresses, in this study, *ZmSPK1* was subcloned into the plant expression vector pGreen0029 under control of the cauliflower mosaic virus 35S promoter and transferred into *Arabidopsis* by *Agrobacterium tumefaciens*. When treated with salt, compared to wild type, transgenic *Arabidopsis* grow better, have higher seedling fresh weight (FW) and dry weight (DW), proline content and superoxide dismutase (SOD) activity; whereas, malondialdehyde (MDA) content and the relative electric conductivity of transgenic plants are kept to a relative lower level. Our results suggest that *ZmSPK1* may play an important role in salt resistance.

Keywords: ZmSPK1; Salt resistance; Transgenic *Arabidopsis*; SOD activity; Proline content.
Abbreviations: ABA-abscisic acid; SOD- superoxide dismutase; MDA- malondialdehyde; FW-fresh weight; DW-dry weight ; SnRK2-sucrose non-fermenting-1-related protein kinase 2; CDPK-Calcium-dependent protein kinase; SNF1-sucrose non-fermenting1; AMPK- AMP-activated protein kinase; WT-wild type; RPK-receptor protein kinase; MAPK- mitogen-activated protein kinase.

Introduction

During growth and development, plants often encounter various environmental stresses, including nutrient deficiency, pathogens, high or low temperature, drought and salinity. Among all these stresses, salinity becomes more and more serious. For example, almost a half of the irrigated agricultural land is affected by high soil salinity (Zhu, 2001). High salt may cause ionic, osmotic, and oxidative stress. Lower osmotic potential could result in the difficulty of water absorption and so on (Zhu, 2002; Leshem et al., 2007). To survive and complete the life circle under those stresses, plants have developed many complex mechanisms perceiving external signals and manifesting adaptive responses (Bohnert et al., 2006; Fujita et al., 2006; Zhu, 2002). In these events, reversible protein phosphorylation catalyzed by protein kinases and protein phosphatases plays a central role (Yang et al., 1997).

To date, numerous protein kinases were proved to be involved in salt stress. For example, The mitogen-activated protein kinase (MAPK) cascade composed of MAPK, MAPKK and MAPKKK is one of the well-known signal transduction factors activated by salinity (Ichimura et al., 2000; Huang et al., 2000). Receptor-like kinases (RLKs) were also found to be induced by salt and other stress signals in *Arabidopsis* (Hong et al., 1997; Osakabe et al., 2005). CDPKs currently known only in plants and protozoa are thought to be one of the key pathways involving in perceiving and transuding the stress-induced calcium signals. It plays important roles in signaling pathways in response to stresses such as drought, salt and cold (Urao et al., 1994; Saijo et al., 2000; Sanders et al., 2002; Ludwig et al., 2004). SNF1 protein kinase family belongs to the CDPK-SnRK superfamily currently comprising SNF-1s in *Saccharomyces cerevisiae*, AMPKs in mammals, and the SnRKs in higher plants (Halford and Hardie 1998; Harmon, 2003). Mammalian AMPKs are considered as a key regulator of cellular and whole body energy homeostasis and can be activated by a lot of pathological and physiological stimuli controlling cellular glucose level and/or AMP: ATP ratio (Hardie et al., 1998). Besides these, AMPKs were recently found to regulate the cell proliferation and polarity indicating their important roles in Mammalian cells (Williams and Brenman 2008). The yeast SNF1s also play important roles in sugar signaling transduction pathway and metabolic stress responses (Hardie et al., 1998). Moreover, SNF1s can also participate in sporulation, glycogen accumulation and peroxisome biogenesis (Hardy et al., 1994; Simon et al., 1992).

Plant SnRKs are grouped into three subfamilies including SnRK1, SnRK2 and SnRK3 according to the sequence similarity and gene structure. SnRK1 is the closest homologue of the yeast SNF1 and the mammalian AMPK with the similar roles in global regulation of carbon and nitrogen metabolism (Halford and Hardie 1998). SnRK2 and SnRK3 subfamilies are unique to plants with less sequence as well as biological functions similarities to SNF1 and AMPK than SnRK1. Recent studies have shown that SnRK2 and SnRK3 mainly function in stress signaling (Hrabak et al., 2003). SOS2, as a well known member of SnRK3 subfamily, is involved in response to salt stress and ABA signaling (Halfter et al., 2000; Liu et al., 2000). To date, a large number of *SnRK2* gene family sequences are found in databases. Kobayashi had identified 10 SnRK2 protein

kinases encoded by the rice genome and found that all family members are activated by hyperosmotic stress and that three of them are also activated by ABA. Among them, *SAPK2*、*SAPK4*、*SAPK6* and *SAPK7* can be induced by NaCl (Kobayashi et al., 2004). Overexpression of *SAPK4* significantly enhances tolerance to salt in rice (Diedhiou et al., 2008). In *Arabidopsis*, 10 SnRK2s have been identified, among which 5 members are activated by ABA, 9 members are activated by hyperosmotic and salinity stresses, whereas none is activated by cold stress (Boudsocq et al., 2004, 2007). In maize 10 SnRK2 members had also been identified, and most *ZmSnRK2* genes are induced by one or more abiotic stress treatments (Huai et al., 2008). *PKABA1* is the first cloned SnRK2 subfamily member in wheat (Anderberg et al., 1992). After that, *TaSnRK2.4* and *TaSnRK2.8* were cloned from wheat, respectively. *TaSnRK2.4* was found to express strongly in booting spindles and to be induced by multi-stresses and ABA application. Overexpression of *TaSnRK2.4* in *Arabidopsis* results in delayed seedling establishment, longer primary roots and enhanced tolerance to abiotic stresses (Mao et al., 2010). In their recent work, overexpression of *TaSnRK2.8* in *Arabidopsis* significantly increases tolerance to drought, salt and cold stresses (Zhang et al., 2010). All these above indicate that *SnRK2* genes play crucial roles in abiotic stress response, and might be involved in diverse developmental processes in plants. In previous study, we have cloned a maize *SnRK2* subfamily gene from maize and named it as *ZmSPK1* (GenBank accession No, AY722708). ZmSPK1 has a kinase activity *in vitro* and can be induced by mannitol, salt and ABA (Zou et al., 2006 a, 2009), which indicate that *ZmSPK1* may be responsible for abiotic stresses. Here, we created the *ZmSPK1* transgenic *Arabidopsis* to further investigate its function in salt stresses. The result shows that overexpression of *ZmSPK1* in *Arabidopsis thaiana* could significantly increase tolerance to salt.

Results

Molecular characterization of transgenic plants

25 individual kanamycin resistant plants were obtained from T_0 seeds after transformation. The kanamycin-resistant T_1 plants were transferred into pots, after that an initial PCR from T_1 plant DNA templates confirmed that most of the kanamycin-resistant plants possess the transformed *ZmSPK1* gene (Figure.2). Only the PCR positive T_1 plants were allowed to set and chosen T_3 homozygous transformants seeds. The chosen homozygous lines were used for immunoblot analysis for further confirmation. As shown in Figure 3, all the PCR positive plants had a specific HA-reactive band with the molecular mass of about 43 kD which was similar to the estimated molecular mass of ZmSPK1. This indicates that the PCR positive plants were transgenic plants and ZmSPK1-dHA fusion protein had been successfully expressed in transgenic plants.

ZmSPK1 enhances salt tolerance in transgenic lines

To examine the roles of *ZmSPK1* in plant salt stress responses, two transgenic lines (L1, L2) and WT plants were treated with NaCl on MS agar plates and in pots. During growing on MS agar plate plus NaCl, the WT plants gradually wilted and finally died, whereas transgenic plants showed higher salt resistance (Figure 4A). To obtain more evidences, the same two transgenic lines were chosen for further salt stress analysis in pots under 300 mM NaCl treatment. 14 days later, the plants were photographed (Figure 4B). After salt treatment, almost all

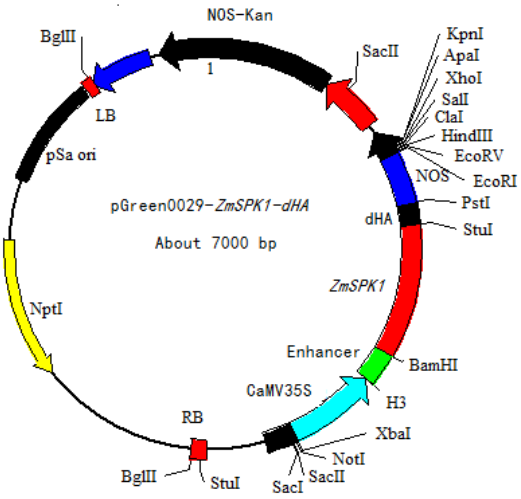

Fig 1. Construction of transformation vector pGreen0029-*35S-C4DDPK-ZmSPK1-dHA-NOS*.

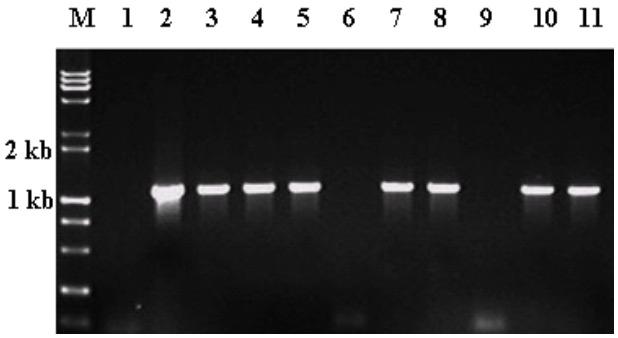

Fig 2. PCR analysis of T_1 transgenic seedlings; M, DNA Marker; 1, WT; 2, plasmid contains *ZmSPK1* cDNA; 3-11, different lines resistance to kanamycin

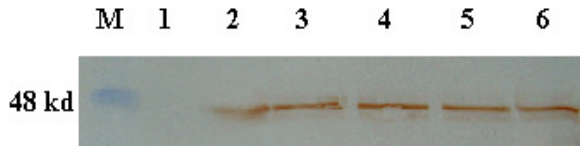

Fig 3. Western-blot analysis of the *ZmSPK1* gene expression in wild type *Arabidopsis* plants and *ZmSPK1* homozygous transgenic lines; M, protein molecular weight marker; WT, wild type *Arabidopsis*; 2~6 different transgenic lines.

the transgenic plants kept alive, 76% L1 plants and 78% L2 plants could flower and set seeds, whereas some of the WT plants had died and only 34% could flower and set seeds. This suggests that the transgenic plants are much more tolerant than the control.

Overexpression of *ZmSPK1* confers increased fresh weight and dry weight under salt treatment

WT and two transgenic lines (L1 and L2) plants were cultivated under salt stress or normal condition as described in materials and methods for FW and DW and the following

physiological measurements. As shown in Figure 5, under normal condition, there are no significant differences between transgenic and WT lines. Under salt stress, FW of L1 and L2 seedling was 0.24 and 0.22 g per plant, respectively, significantly higher than that of WT plants (Figure 5A). Similarly, DW of L1 and L2 seedling was 21.7 mg and 19.6 mg per plant, respectively, significantly higher than that of WT plants (Figure 5B). This result shows that overexpression of *ZmSPKE* enhances fresh and dry weight under salt treatment in transgenic *Arabidopsis.*

Physiological characterization of transgenic plants

In order to examine the physiological traits related to plant salt stress tolerance, SOD activity, ion leakage ratio, MDA and proline content were analyzed in *ZmSPK1* transgenic lines. Under normal condition, SOD activity of transgenic plants had no significant differences with that of WT plants. When treated with salt solution, SOD activities of both transgenic and WT plants increased sharply at the beginning and then decreased gradually. During the treatment period, SOD activity of transgenic plants was significantly higher than that of WT plants (Fig. 6A). This shows that overexpression of *ZmSPK1* can increase SOD activity under salt treatment. Proline is an osmoprotecting molecule, which accumulates in response to water stress and salinity (Claussen W, 2005; Younis ME et al., 2009). As shown in Figure 6B, under normal condition, there was no difference in proline content between WT and transgenic plants. When treated with salt solution, proline contents in both transgenic and WT plants increased rapidly to higher levels. Moreover, proline contents in transgenic plants increased more rapidly than that of WT plants and significantly higher than that of WT plants during all the treatment time. This shows that overexpression of *ZmSPK1* can increase proline content under salt treatment. MDA is the product of lipid peroxidation, its content in plants reflects the degree of cell injury (Nagesh BR et al., 2008; Pérez-Tornero et al., 2009). As shown in Figure 6C, there was no difference in MDA content between WT and transgenic plants under normal condition and at the beginning of salt treatment. While, after the 2nd day of treatment MDA contents in both transgenic and WT plants increased rapidly to higher levels. Furthermore, MDA contents in WT plants increased more rapidly than that of transgenic plants and significantly higher than that of transgenic plants between 4-6 days of treatment. This shows that overexpression of *ZmSPK1* can decrease MDA content thus decreasing the injury of plants under salt stress. Ion leakage ratio also reflects the degree of plant cell injury under stress treatment. Similarly, there was no obvious difference among the ion leakage ratio of the tested plants under normal condition (Figure 6D). When treated with salt solution, ion leakage ratio in both transgenic and WT plants increased rapidly to higher levels. Between 2-6 days of salt treatment, ion leakage ratio of transgenic plants was significantly lower than that of WT plants. This indicates that overexpression of *ZmSPK1* can protect the cell membrane under salt treatment.

Discussion

It has been demonstrated that *SnRK2* genes are involved in response to multi-environmental stresses (Umezawa et al., 2004; Kobayashi et al., 2005; Zhang et al., 2010). Our work suggested that *ZmSPK1* was also induced by mannitol, salt and ABA (Zou et al., 2006 a). So, our hypothesis is that overexpression of *ZmSPK1* gene might enhance some abiotic stresses tolerance, such as cold, drought and salt. However, when treated with cold and drought the transgenic plants did not exhibit enhanced tolerance than WT plants (data not shown). The ABA content in transgenic lines and WT plants, as well as the effects of exogenous ABA application on seed germination of transgenic lines and WT on MS medium were measured, but no obvious differences were indentified (data not shown), showing that there may be no direct relationship between ABA and ZmSPK1. All these above indicate that *ZmSPK1* might mainly participate in salt stress, supporting our previous opinion (Zou et al., 2006 a). In this study, experiments were performed for the evaluation of salt tolerance between the transgenic lines and WT plants based on both morphological and physiological characteristics. On MS agar plates supplemented with 200 mM NaCl, WT plants displayed growth inhibition and almost all died after 1 week of salt treatment. Meanwhile, leaves of transgenic plants still kept green at this time, although they also displayed growth inhibition to some degree. Furthermore, when treated with NaCl solution, transgenic plants exhibited higher survival ration and biomass than WT plants. These results show that *ZmSPK1* might play an important role in salt resistance pathway. High salt may not only lead to ionic and osmotic stress but also cause oxidative stress because of the induction of generating reactive oxygen species (ROS) (Zhu, 2002; Leshem et al., 2007). This oxidative stress can lead to lipid peroxidation and membrane permeabilization and as a result, ion leakage ratio and MDA content in the living cells increase (Scandalios, 1993; Pérez-Tornero et al., 2009). SOD is a key enzyme to alleviate the oxidative damage by removing the reactive oxygen

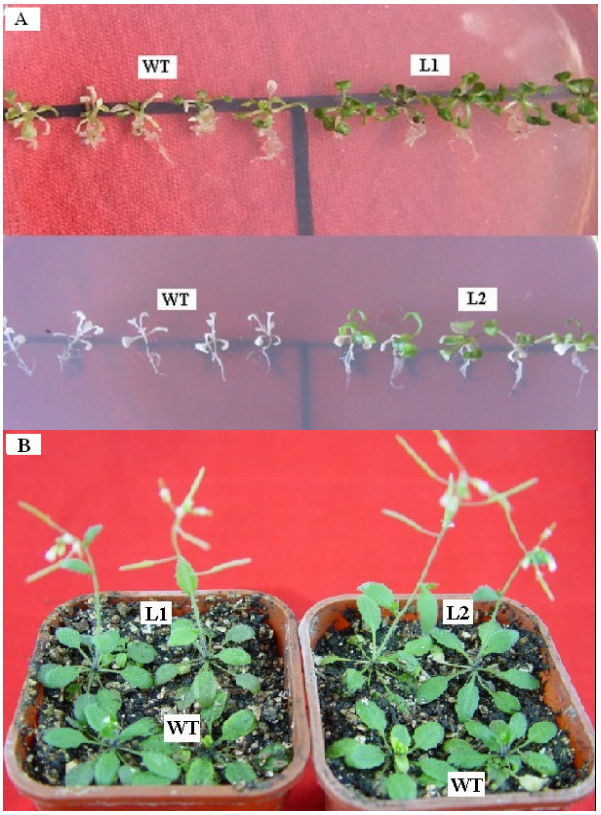

Fig 4. Comparison of WT *Arabidopsis* plants and two transgenic lines (L1, L2) under salt treatments. A, Comparison of WT plants and transgenic lines growing on MS agar plates plus 200 mM NaCl. B, Comparison of the WT plants and transgenic lines growing in pots under 300 mM NaCl solution treatment.

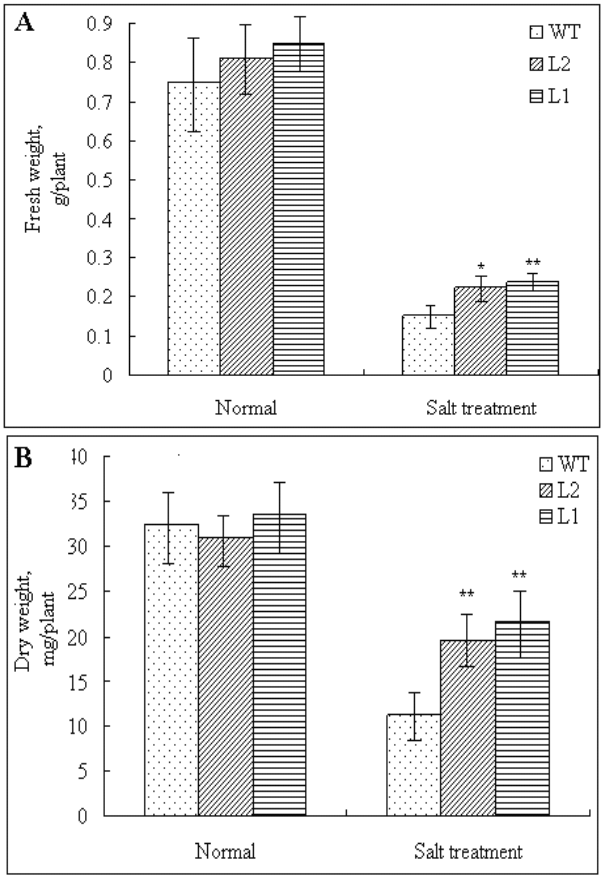

Fig 5. FW (A) and DW (B) of WT and transgenic plants after normal or salt treatment. Error bars indicate ±SE (n = 3). * and **, Significantly different from the WT at $P < 0.05$ and < 0.01, respectively, by Student's *t* test.

intermediates (ROIs) (Sairam et al., 2005; Moussa HR et al., 2008); A few studies showed a positive correlation between SOD activity level and salt tolerance (Rout and Shaw, 2001). It was also reported that transgenic plants overexpressing *SOD* gene led to enhanced salt tolerance (Wang et al., 2010). Proline is also considered as a typical physiological parameter for evaluating salt stress tolerance and resistance in crop plants. When encountered with external environmental stresses many plants decrease their cellular osmotic potentials through accumulation of intracellular organic osmolytes such as proline to maintain a stable intracellular environment (Zhu, 2002; Granier et al., 1999; Wang et al., 2007). In this study, under salt treatment, SOD activity and proline content in transgenic plants were significantly higher than that in WT plants, whereas, ion leakage and MDA content in transgenic plants were significantly lower than that in WT plants. This indicated that *ZmSPK1* might enhance salt tolerance by increasing the capability of reactive oxygen alleviation and osmotic adjustment.

Materials and methods

Construction of plant expression vector and transformation

The sequence of *ZmSPK1* gene with *dHA* tag (*ZmSPK1-dHA*) has been successfully inserted into a yeast expression vector p426GAL1 in previous work (Zou et al., 2009). The sequence

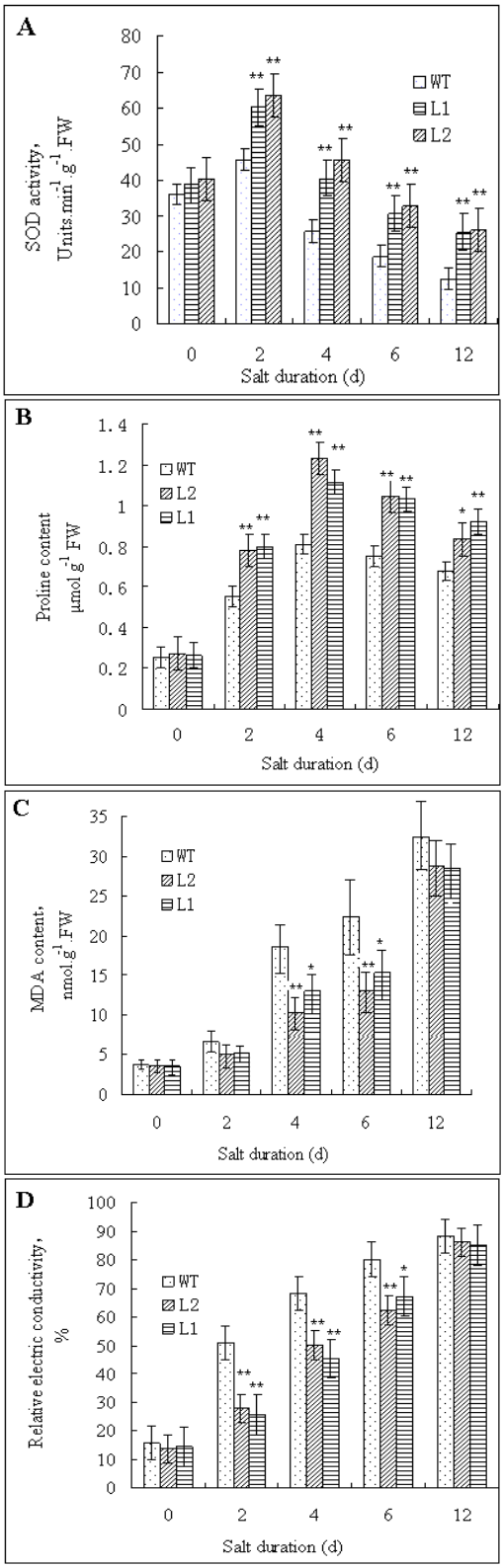

Fig 6. Physiological characterizations of WT and transgenic plants. (A) SOD activity, (B) Proline content, (C) MDA content, (D) ion leakage ratio. Error bars indicate ±SE (n = 3). * and **, Significantly different from the WT at $P < 0.05$, and < 0.01, respectively, by Student's *t* test.

of *ZmSPK1-dHA* in recombinant plasmid p426GAL1 was amplified by PCR with adding *Bam*H I / *Pst* I sites into 5'and 3' primers, respectively. The PCR product was then digested with *Bam*H I / *Pst* I. The recombinant plasmid pGreen0029-*35S-C4DDPK-CBF3-NOS* (stored in our lab) was also digested with *Bam*H I / *Pst* I to release the *CBF3*. The digested products including the sequence of *ZmSPK1-dHA* and the pGreen0029 vector fragment were ligated with T4 DNA ligase (TaKaRa, Dalian, China) to produce the recombinant plasmid pGreen0029-*35S-C4DDPK-ZmSPK1-dHA-NOS*. (Figure 1). HA refers to human influenza hemagglutinin, dHA tag (YPYDVPDYAGYPYDVPDYA) are used as a general epitope tag in expression vectors, which does not appear to interfere with the bioactivity or the biodistribution of the recombinant protein. The tag facilitates the detection of recombinant proteins by Western-blot analyses. After sequencing for verification, the recombinant plasmid pGreen0029-*35S-C4DDPK-ZmSPK1-dHA-NO* was introduced into *Agrobacterium tumefaciens* strain GV3101 which was then used to transform *Arabidopsis* (ecotype Columbia) using the floral dip method (Clough and Bent, 1998).

Plant material, growth conditions and stress treatments

Plant material, growth conditions and salt treatments were performed as described by Zou et al. (Zou et al., 2010).

Molecular characterization of transformants

The resistant seedlings growing on kanamycin medium were transferred to soil in the plastic pots. When the seedlings grew enough, 2-3 leaves were cut for extraction of genome DNA (Zou et al., 2006 b). A pair of primers specific to Z*mSPK1* was used to amplify by PCR. About 300 mg leaves from wild-type and transgenic plants were ground in liquid nitrogen and homogenized in extraction buffer containing 10 mmol·L^{-1} Tris-HCl, pH 8.0, 0.02% NaN_3, 0.5 mM phenylmethylsulfony fluoride. After centrifugation at 16,000 g for 10 min, aliquots of supernatant were stored at -80°C. Western-blot was conducted according to the protocol of Zou et al (Zou et al., 2009).

Physiological assays

Measurement of SOD activity, proline and MDA content and relative electric conductivity was conducted at 0, 2, 4, 6, 12 d, respectively, after salt treatment. Total SOD activity was assayed according to the method of Jiang et al. (Jiang and Zhang, 2002). Proline content was determined according to Tijen and Ìsmail (Tijen and Ìsmail, 2005). To measure ion leakage ratio as relative electric conductivity parameter, 0.1 g same positional leaves were removed from different plants, rinsed briefly with deionized water and immediately placed into a tube with 10 mL of deionized water. Conductivity (I_1) was measured using an electroconductivity meter (model 1054, VWR Scientific, Phoenix) after the tubes were placed at 22°C overnight. Then, the samples were heated at 100°C for 30 min and conductivity (I_2) was measured again. Ion leakage ratio was expressed as (I_1/I_2) ×100%. For all the above measurements, three replicates per line were used. Statistical differences were determined using Student's two-tailed t test.

Acknowledgements

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