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ZmPto, a maize *Pto*-like gene, significantly affects salt resistance in transgenic *Arabidopsis*

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Abstract

Studies have shown that Pto (confers resistance to *Pseudomonas syringae* pv. *tomato*) plays an important role in plant disease resistance pathway. However, little is known about its responses to salt stress. A previous study has shown that maize *ZmPto* (*Pto*-like gene from *Zea mays* L.) is induced by salicylic acid (SA), abscisic acid (ABA), mannitol and salt. In this study, *ZmPto* was over-expressed in *Arabidopsis* in order to further analyze its biological functions. Under salt stress, transgenic plants grew better, had significantly higher seedling fresh and dry weight (FW, DW), seed yields, proline content and lower relative electric conductivity level compared with wild type. The seedling FW of transgenic lines, L1 and L2, increased by 40% and 60%, respectively. In addition, the DW of transgenic lines seedling, L1 and L2, increased 45% and 53%, respectively. The seed weight of L1 and L2 transgenic lines increased 73 % and 120 %, respectively. Semi-quantitative RT-PCR revealed that compared with the wide type (WT) plants, the expression levels of *RD29B*, *KIN2*, *SOS3* and *P5CS1* in transgenic lines increased significantly. To our knowledge, this is the first report from the effect of over-expression of Pto-like gene which enhances the salt resistance in plants.

Keywords: ZmPto; Transgenic *Arabidopsis*; Salt resistance; Seed yields; Proline content.
Abbreviations: ABA- abscisic acid; SA- salicylic acid; FW- fresh weitht; DW- dry weight; WT- wild type; ROS- reactive oxygen species; Pti1- Pto-interacting1; CDPK- Calcium-dependent protein kinase; MAPK- mitogen activated protein kinases; SOS- salt overly sensitive; DEX- dexamethasone

Introduction

Salinity is one of the most severe threats to crop yield worldwide and becoming more and more serious. For example, a vast part of our world's arable land and almost a half of the irrigated agricultural land is affected by high soil salinity (Zhu, 2001). High salt in the soil leads to ionic, osmotic, and oxidative stress, seriously affecting plant growth and development (Zhu, 2002). High salt could also lower the osmotic potential and; therefore, a restricted uptake of water occurs. Additionally, salt stress can cause oxidative stress because of the induction of reactive oxygen species (ROS) (Leshem et al., 2007). To survive, plants have formed their protection events to acclimate to the environmental stresses (Pastori et al., 2002). In these events, reversible protein phosphorylation catalyzed by protein kinases and protein phosphatases plays a key role (Yang et al., 1997). Many protein kinases, such as mitogen activated protein (MAP) kinases, calcium-dependent protein kinases (CDPKs), receptor-like kinases, and histidine kinases have been proved to be involved in salt resistance signal pathway (Xiong et al., 2002; Zhu, 2002; Shinozaki et al., 2003; Lingkun et al., 2010; Takayuki et al., 2011). The salt overly sensitive (SOS) is also a well known signaling pathway for resistance to salt stress (Zhu, 2002; Qing et al., 2009). In this pathway, SOS2 encodes a serine/threonine protein kinase (Liu et al., 2000). As a calcium sensor, SOS3 encodes an EF-hand Ca^{2+}-binding protein playing important roles in salt resistance signal pathway (Liu and Zhu, 1998). Salt-elicited Ca^{2+} signal can be sensed by SOS3 which in turn is activated by SOS2 (Halfter et al., 2000). Then the SOS2/SOS3 kinase complex phosphorylates and activates SOS1. As a plasmamembrane Na^+/H^+ antiporter, SOS1 plays a central role in sodium extrusion and controlling long distance Na^+ transport (Qiu et al., 2004; Shi et al., 2000, 2002).

The Pto gene, which encodes an intracellular Ser/Thr protein kinase, was isolated in 1993 by map-based cloning from tomato and was the first recognition resistance (R) gene cloned from *planta* (Martin et al., 1993). Over-expression of Pto gene in tomato results in a broad-spectrum resistance, not only to *Pseudomonas* strains but also to many other bacterial and fungal pathogens (Tang et al., 1999). In the Pto resistance pathway, the Pto protein directly interacts with either of the two pathogen effector proteins, AvrPto or AvrPtoB from *Pseudomonas syringae* pv *tomato* resulting in the HR-mediated resistance (Martin et al., 1993; Scofield et al., 1996; Kim et al., 2002). Pto-interacting (Pti) proteins are proved to be downstream components of Pto by yeast two-hybrid system. One of these, Pti1, also a serine/threonine protein kinase, is specifically phosphorylated by Pto. Overexpression of Pti1 enhances Pto-mediated cell death in tobacco (Zhou et al., 1995). Other three, Pti4, Pti5 and Pti6, are transcription factors. Overexpression of these proteins in *Arabidopsis* enhances expression of SA-mediated PR genes and also resistance to *Pseudomonas syringae* pv *tomato* and a fungal pathogen (Zhou et al., 1997; Gu et al., 2002). To date, all the reports about Pto gene are disease resistance related. There is no report about Pto gene involving in salt resistance. In previous study, a Pto-like kinase gene was cloned from maize named *ZmPto*. Yeast two-hybrid analysis showed the ZmPto can interact with ZmPti1. RT-PCR analysis indicated that the *ZmPto* expression is induced by SA, ABA, mannitol and salt, suggesting its important roles in both biotic and abiotic stresses (Zou et al., 2011 b). The main goal of this study is to confirm the biological function of *ZmPto*. The results showed that overexpression of *ZmPto* in *Arabidopsis* enhance salt resistance, but has no obvious effect on disease resistance.

Results

Molecular analysis of transgenic lines

After transformation, 22 individual hygromycin resistance plants were obtained from T_0 seeds. The hygromycin-resistant T_1 plants were transferred into pots, and then characterized by PCR. Results showed that most of the hygromycin-resistant plants are PCR positive (data not shown). Only the PCR positive T_1 plants were allowed to set and choose T_3 homozygous transformants seeds. The chosen homozygous lines were further confirmed via immunoblot analysis. As shown in Fig. 1, all the PCR positive plants have a specific HA-reactive band with the molecular mass of about 45 KD, similar to the estimated molecular mass of ZmPto. This not only proves that the PCR positive plants are transgenic plants, but also proves that the ZmPto-dHA fusion protein has been successfully expressed in transgenic plants.

Salt tolerance characterization of transgenic Arabidopsis

Previous study indicated that *ZmPto* can be induced by salt stress. So it is interesting to check whether salt stress can cause any phenotypic change in the *ZmPto* transgenic plants. Two transgenic lines (L1, L2), as well as the WT controls, were used for the examination of salt tolerance. When treated with 200 mM NaCl, the germination of the WT seeds were almost completely inhibited, while the two transgenic lines still had a significantly higher germination rate (Fig. 2A). To confirm the salt resistance of the transgenic plants, the same two transgenic lines were chosen for further salt treatment in pots under 300 mM NaCl treatment. 2 weeks after treatment, the plants were photographed (Fig. 2B). After salt treatment, the growth of the two transgenic and WT plants is retarded. However, the two transgenic lines grew better than the WT plants. Most of the transgenic plants were alive, 61% of L1 and 72% of L2 plants could produce flowers and set seeds, whereas most of the WT plants have died and only 26% could set flower and seeds. In addition, both transgenic lines showed earlier flowering property than the WT plants under high salt conditions, suggesting that transgenic plants have more salt resistant phenotype than the wile type.

Morphological and Physiological responses of transgenic Arabidopsis under salt treatment

Both fresh and dry weight in transgenic lines did not statistically differ from wild type plants under normal conditions. Salt stress, as expected, resulted in a significant decline in fresh and dry weight of WT. Seed weight is often considered as the representative factor of economic yields under stress treatment. Under normal condition, seed weight of L1, L2 plants were 25.12 and 24.33 mg per plant, respectively, slightly lower than that of WT plants, which was 26.21 mg per plant. When treated with 300 mM NaCl, seed weight of L1, L2 plants were 9.01 and 11.46 mg per plant, respectively, significantly higher than that of WT plant (Table 1). Electrolyte leakage is often used to provide an estimate of membrane injury under stress treatment. As shown in Table 2, there is no obvious difference among the ion leakage ratio of the tested plants under normal condition. When treated with salt, ion leakage ratio in both transgenic and WT plants increased to higher levels, indicating that membrane damage had been caused by salt stress. On the other hand, between 1-6 days of salt treatment, ion leakage ratio of transgenic plants was significantly lower than that of WT plants. This result indicates that over-expression of *ZmPto* can stabilize the cell membrane more than the WT plants for a relatively long period under salt stress treatment. The proline accumulation facilitates the cell tolerance to water stress and high salinity (Claussen, 2005; Younis et al., 2009; Gao et al., 2011). The analysis of proline content revealed that there is no statistical significance between transgenic and wild type plants under normal condition. After salt treatment, the proline content increased in both transgenic and wild-type plants. Moreover, the proline content in the transgenic plants was significant higher than that in the WT plants during all the treatment period (Table 2). This shows that over-expression of *ZmSPK1* can increase proline content under salt treatment.

Expression of stress-related genes in transgenic Arabidopsis

To evaluate the implications of *ZmPto* gene in stress response pathways, the expression levels of 5 genes, *ADH1*, *RD29B*, *KIN2*, *SOS3* and *P5CS1*, all closely associated with plant salt tolerance, were measured under non-salt-stress conditions by semi-quantitative RT-PCR. From Fig. 3, we can see that the transgenic plants have higher transcript levels of *RD29B*, *KIN2*, *SOS3* and *P5CS1* compared to WT plants. Among them *RD29B* had the most significant increase in transgenic plants, approximately 3 to 8-fold higher than that in the wild-type plants. The expression of *A*DH1 remains approximately the same.

Discussion

ZmPto affects germination and seedling growth under salt treatment

Our previous study showed that *ZmPti1* also play main roles not only in disease but in salt resistance pathway in transgenic *Arabidopsis*. These indicate that *Pto/Pti1* signal pathway in maize might mainly participate in salt stress, suggesting the complexity of the *Pto/Pti1* signal pathway in *planta*. In this study, experiments were performed for the evaluation of salt tolerance between the transgenic and WT plants via morphological and physiological characterizations. On MS agar plates supplemented with 200 mM NaCl, germination of WT seeds are almost entirely inhibited, while some of the transgenic seeds could still geminate, although they also displayed germination inhibition to some degree. Biomass and economic yields are useful traits to evaluate the stress tolerance. In this study, when treated with NaCl solution, transgenic plants exhibit higher survival rate, biomass and yields than WT plants. These indicate that *ZmPto* might play an important role in salt resistance.

ZmPto affects physiological traits of transgenic Arabidopsis under salt treatment

To explore the mechanisms underlying the possible *ZmPto*-mediated regulation of salt tolerance, some physiological traits related to plant salt tolerance were measured in this study. Relative electric conductivity is an important indicator for cell membrane injury, resulting from the oxidative stress under stresses (Holmberg et al., 1998; Hasegawa et al., 2000; Mittova et al., 2004; Pérez-Tornero et al., 2009). Under salt stress, *ZmPto*-overexpressing *Arabidopsis* contain a lower ion leakage ratio compared with the WT plants, indicating that *ZmPto* might enhance salt tolerance by reducing the injury of cells under salt stress. Proline is commonly present as an organic solute in plants. It is well known that proline accumulation can increase the osmotic pressure and, thus, improve the salt tolerance of plants (Armengaud et al., 2004). In our study, the proline content in transgenic plants is higher than that in the WT plants under salt treatment. This result is consistent with the expression level of *P5CS1*, a major rate-limiting enzyme in proline synthesis (Delauney et al., 1990; Hu et al., 1992). These data indicated that Zm*Pto* migh increase the expression of *P5CS1* gene and, subsequently, enhance the proline accumulation for osmotic adjustment, thus improving the salt tolerance of the transgenic plants.

Table 1. Yield, FW and DW of WT and transgenic plants under normal and salt stress conditions.

Plant line	Fresh weight (g)		Dry weight (mg)		Yield (mg)	
	Normal condition	Salt treatment	Normal condition	Salt treatment	Normal condition	Salt treatment
WT	0.55	0.15	30.12	15.25	26.21	5.22
L1	0.52 ^{ns}	0.21 *	27.52 ^{ns}	22.10 **	25.12 ^{ns}	9.01 **
L2	0.56 ^{ns}	0.24 **	26.14 ^{ns}	23.26 **	24.33 ^{ns}	11.46 **

Data represent the means ±SE of three experimental replicates; * and **, significantly different from the WT at $P < 0.05$ and < 0.01, respectively, by Student's t test, ns means non-significant.

Table 2. Ion leakage ratio and proline content of of WT and transgenic plants under normal and salt stress conditions.

Plant line	Relative electric conductivity (%)					Proline content (μmol/g.FW)				
	0 day	1 day	3 days	6 days	10 days	0 day	1 day	3 days	6 days	10 days
WT	14.67	40.87	58.23	70.12	78.21	0.224	0.503	0.762	0.704	0.728
L1	13.32 ^{ns}	28.46 **	45.34 **	62.23 *	7 5.23 ^{ns}	0.282 ^{ns}	0.733 *	9.981 **	8.145 *	0.886 *
L2	12.56 ^{ns}	22.67 **	40.12 **	52.34 **	76.13 ^{ns}	0.314 ^{ns}	0.751 *	1.265 **	1.032 **	1.073 **

Data represent the means ±SE of three experimental replicates; * and **, significantly different from the WT at $P < 0.05$ and < 0.01, respectively, by Student's t test, ns means non-significant.

ZmPto affects diverse downstream genes

To elucidate the molecular mechanism of *ZmPto* in response to salt, we analyzed the expression levels of 5 salt stress-related genes. *RD29B* and *KIN2* have been used as convenient markers for monitoring the ABA and stress response pathways in plants because of ABA-responsive element in their promoter region (Pandey et al. 2004). Our data showed that transgenic plants increase the expressions of *RD29B* and *KIN2* transcripts comparing with WT plants under salt condition, suggesting that *ZmPto* may be involved in plant stress response, probably in an ABA-dependent manner. This result is in agreement with our previous report (Zou et al., 2011 b). Alcohol dehydrogenase gene ADH1 is up-regulated by ABA and stresses. But in our study, the expression of ADH1 has no obvious change in transgenic plants compared with WT plants, indicating that ADH1 pathway may not play main roles in *ZmPto* signal pathway. SOS system is a well known salt resistance pathway, in which SOS3 plays a pivotal role (Zhu, 2002; Qing et al., 2009). In this study, the expression of *SOS3* gene also increased in transgenic lines. This shows that ZmPto may also have a cross-talk with SOS pathway. However, the detailed mechanisms are still unknown and need to be studied further.

Materials and methods

Expression vector construction and Arabidopsis transformation

The DEX-inducible promoter vector pTA7002 was provided by Dr. Nam-Hai Chua (Rockefeller University, New York). In this study, the over-expressed gene was inserted at the *Xho*I and *Spe*I restriction sites as recommended by Dr. Chua. The segment *ZmPto-dHA* in the expression vector pGreen0029 (unpublished) was amplified by PCR with adding *Sma*I and *Spe*I sites into 5' and 3' primers, respectively, and then digested with *Sma*I / *Spe*I. The pTA7002 plasmid was first digested with *Xho*I and then blunted with DNA blunting kit (TaKaRa, Dalian, China). The following construct was digested with *Spe*I once again. Finally the digested products including the sequence of *ZmPto-dHA* and the pTA7002 vector fragment were ligated with T_4 DNA ligase (TaKaRa, Dalian, China) to produce the recombinant plasmid. The resulting construct 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). The T_0 seedlings were screened by 30 mg L^{-1} hygromycin medium. Resistant seedlings were transferred to soil in the plastic pots and grown to produce seeds. The T_1 seeds were subsequently selected until T_3 homozygous seeds were obtained.

Plant material, growth conditions

Plant material, growth conditions were performed as described by Zou et al. (Zou et al., 2011 a).

Molecular characterization of transformants

PCR characterization of transformants was performed as described by Zou et al. (Zou et al., 2011 a) except that the primers specific to ZmPto was used here to amplify by PCR. Before Western-blot analysis, wild-type and the PCR positive transgenic plants grown in growth chamber were sprayed with10 μM DEX solution. 24 h after treatment, about 300 mg leaves from wild-type and transgenic plants were harvested for Western-blot analysis according to the protocol of Zou et al (Zou et al., 2011 a).

NaCl treatment, morphological and physiological traits measurements

For germination experiment, WT and transgenic homozygous seeds were sowed on agar plates containing 4.4 g/L MS powder medium (Murashige and Skoog basal medium with gamborg's vitamins, Sigma) and 30 g/L surcose supplemented with 200 mM NaCl and 1 μM DEX, 10 days later, plants were photographed. Salt stress experiment was also conduced in growth chambers. At the six true leaves stage, salt treatment was started by watering with 300 mM NaCl until the soil was saturated, the treatment was conducted once every three days. At the same time, all the plants were treated with 10 μM DEX solution every other day. Measurement of proline content and relative electric conductivity was conducted at 0 (normal condition control), 1, 3, 6, 10 d, respectively, after salt and DEX treatments. When visual symptoms (such as the wilting degree, the plant size and so on) appeared plants were harvested for seedling fresh and dry weight. The fresh weight of whole plants was measured immediately after the harvest. Dry weight of whole plants was measured after 48 h at 80°C. When all the treated plants maturated, the seeds of WT and transgenic plants were harvested and weighed.

Statistical analyses

The randomized complete block design was used in FW, DW, yield, relative electric conductivity and proline content assay. Means ±SE were calculated from the data of three replications. Statistical differences were determined using Student's two-tailed t test.

Fig 1. Western-blot analysis of the ZmPto protein from WT plants and *ZmPto* homozygous transgenic lines induced by DEX. M, protein molecular weight marker; WT, wild type *Arabidopsis*; Numbers 1-5, different transgenic lines.

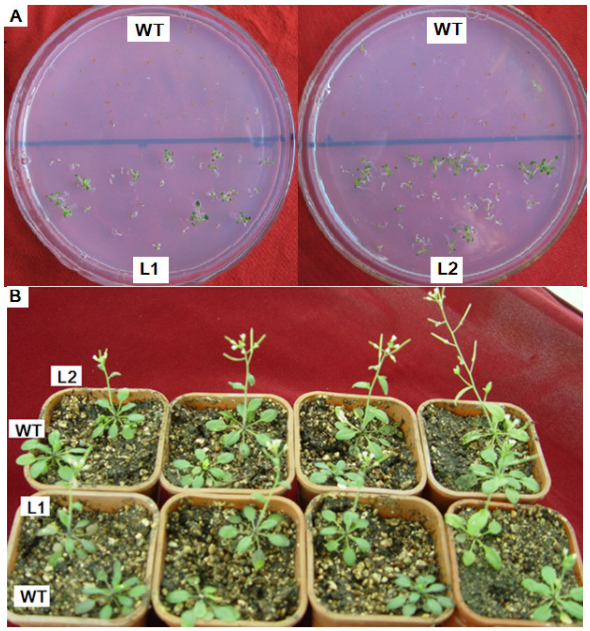

Fig 2. Effects of salt stress on the WT and transgenic plants (L1, L2). (A) Seeds of WT and two transgenic lines were germinated on MS plus 200 mM NaCl. (B) Comparison of the WT plants and transgenic lines growing in pots under 300 mM NaCl solution treatment.

Semi-quantitative RT-PCR characterizations

The WT and transgenic homozygous transgenic plants were grown in pots in growth chambers. At the six true leaves stage, all the plants were sprayed with 10 μM DEX solutions. After 24 h of treatment with DEX, young leaves were harvested for RNA extraction, The RNA extraction and Semi-quantitative RT-PCR procedures were performed as described previously (Zou et al., 2006). Semi-quantitative RT-PCR reactions were performed using the gene-specific primers for stress marker genes (24 cycles for *18S rRNA*, 25 cycles for *ADH1*, *KIN2* and *SOS3*, 26 cycles for *RD29B*, and 28 cycles for *P5CS1*). The primers were designed as follows: *18S rRNA* forward primer, 5'-CCATAAACGATGCCGA-3'; *18S rRNA* reverse primer, 5'-CACCACCCATAGAATCAAGA-3'; *RD29B* forward primer, 5'-GACGAGCAAGACCCAGAAGT-3'; *RD29B* reverse primer, 5'-TGCTCTGTGTAGGTGCTTGG-3'; *ADH1* forward primer, 5' -CTCTTGGTGCTGTTGGTTTAGG-3'; *ADH1* reverse primer, 5'-AATTGGCTTGTCATGGTCTTTC-3'; *KIN2* forward primer, 5'-GTCAGAGACCAACAAGAATGCC-3'; *KIN2* reverse primer, 5'-TGACTCGAATCGCTACTTGTTC-3';

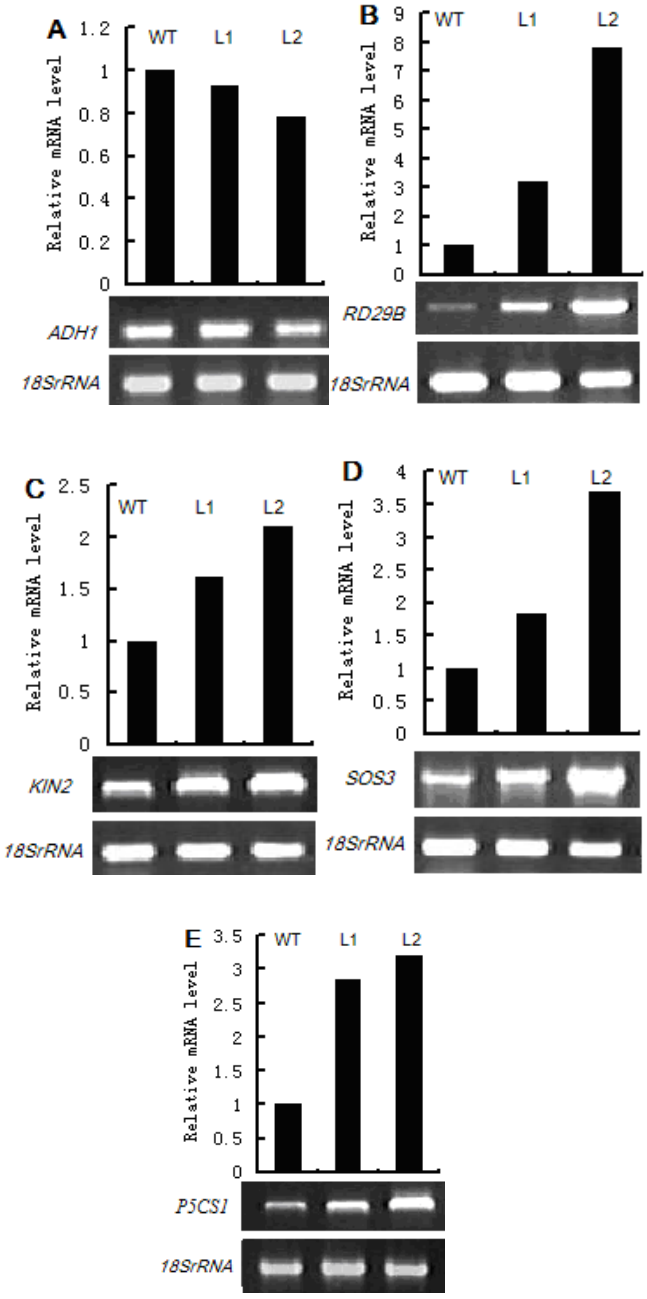

Fig 3. Semi-quantitative RT-PCR analysis of *ADH1* (A), *RD29B* (B), *KIN2* (C), *SOS3* (D), and *P5CS1* gene (E) in WT and transgenic *Arabidopsis*.

SOS3 forward primer, 5'-GGAGGAATCTCTTCGCTG-3';
SOS3 reverse primer 5'-CACGAAAGCCTTATCCACC-3';
P5CS1 forward primer, 5'-GTGGCTCGCTTAGTTATG-3';
*P5CS1*reverse primer, 5'-GGAATGTCCTGATGGGTG-3'.

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