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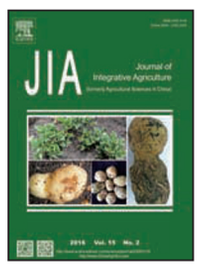

RESEARCH ARTICLE

Overexpression of *IbMIPS*1 gene enhances salt tolerance in transgenic sweetpotato

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Abstract

Myo-inositol-1-phosphate synthase (MIPS) is a key rate limiting enzyme in the *de novo* biosynthesis of *myo*-inositol in plants. In the present study, the *IbMIPS*1 gene was introduced into sweetpotato cultivar Xushu 18 and the transgenic plants exhibited significantly enhanced salt tolerance compared with the wild-type (WT). Overexpression of *IbMIPS*1 up-regulated the salt stress responsive genes, including *myo*-inositol monophosphatase (*MIPP*), pyrroline-5-carboxylate synthase (*P5CS*), pyrroline-5-carboxylate reductase (*P5CR*), *psbA*, phosphoribulokinase (*PRK*), and superoxide dismutase (*SOD*) genes, under salt stress. Inositol and proline content, SOD and photosynthesis activities were significantly increased, whereas malonaldehyde (MDA) and H_2O_2 contents were significantly decreased in the transgenic plants. These findings suggest that the *IbMIPS*1 gene may enhance salt tolerance of sweetpotato by regulating the expression of salt stress responsive genes, increasing the content of inositol and proline and enhancing the activity of photosynthesis.

Keywords: *IbMIPS1*, overexpression, salt tolerance, sweetpotato

1. Introduction

Salt stress is one of the major environmental stresses which affect crop productivity and quality in world agriculture (Munns and Tester 2008; Zhao *et al*. 2013). The development of crops with improved salt tolerance is therefore important. Sweetpotato, *Ipomoea batatas* (L.) Lam., is an important food, industrial and bio-energy resource crop, there is an increased demand for this crop but this goal is often limited by abiotic stresses (Zang *et al*. 2009; Liu *et al*. 2014c). Sweetpotato as source of bio-energy will mainly be planted on marginal land, thus, salt stress is a critical delimiter for the cultivation expansion of this crop. To maintain productivity of sweetpotato on marginal land, it is important to improve its salt tolerance by genetic engineering.

There are several reports on improving salt tolerance by genetic engineering in sweetpotato. A chloroplastic *BADH* gene from spinach was introduced into sweetpotato and the transgenic plants showed enhanced tolerance to salt, methyl violet (MV)-mediated oxidative and low temperature stresses (Fan *et al*. 2012). Overexpression of *IbOr* gene from sweetpotato increased carotenoid accumulation and salt tolerance in transgenic sweetpotato calluses (Kim *et al*. 2013a). The down-regulation of *IbLCY-ε* gene improved carotenoid synthesis and salt tolerance in sweetpotato transgenic calluses (Kim *et al*. 2013b). Wang *et al*. (2013a) isolated *IbNFU*1 gene from sweetpotato and Liu *et al*. (2014c) found this gene was involved in salt tolerance of

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sweetpotato. Overexpression of *IbP5CR*, *IbMas* or *IbSIMT*1 gene enhanced salt tolerance in transgenic sweetpotato (Liu *et al*. 2014a, b, d).

Myo-inositol-1-phosphate synthase (MIPS, EC 5.5.1.4) is a key rate limiting enzyme of *myo*-inositol biosynthesis which catalyzes the reaction from glucose-6-phosphate (G-6-P) to *myo*-inositol-1-phosphate (Ins1P), which is subsequently dephosphorylated by *myo*-inositol monophosphatase (MIPP) to form free inositol (Abreu and Aragão 2007). In plants, inositol is a precursor for many inositol containing compounds and is implicated in various physiological and biochemical processes such as growth regulation, cell membrane biogenesis, hormonal regulation, programmed cell death, stress signalling, plant immunity, etc. (Kaur *et al*. 2013; Tan *et al*. 2013).

The *MIPS* genes have been isolated from several plant species, such as *Arabidopsis* (Johnson 1994), rice (Yoshida *et al*. 1999), maize (Larson and Raboy 1999), tobacco (Hara *et al*. 2000), soybean (Hegeman *et al*. 2001), *Jatropha curcas* (Wang *et al*. 2011), smooth cordgrass (Joshi *et al*. 2013), and *Medicago falcate* (Tan *et al*. 2013). Overexpression of *PcINO*1 (*PcMIPS*) enhanced tolerance to salt in transgenic *Brassica juncea* and rice (Das-Chatterjee *et al*. 2006) and tolerance to salt and oxidative stresses in transgenic *Brassica juncea* and increased the seed survival rate under salt and dehydration stresses in transgenic tobacco plants (Goswami *et al*. 2014). The *CaMIPS*2/*SaINO*1-overexpressing *Arabidopsis* showed enhanced salt tolerance (Kaur *et al*. 2013; Joshi *et al*. 2013). Tan *et al*. (2013) reported that overexpression of *MfMIPS*1 improved resistance to chilling, drought and salt stresses in transgenic tobacco plants.

In our previous study, the *IbMIPS*1 gene was cloned from sweetpotato cultivar Nongda 603 (Zhai and Liu 2009). In the present study, we introduced this gene into an important sweetpotato cultivar Xushu 18 and found that the *IbMIPS*1-overexpressing plants exhibited significantly enhanced salt tolerance.

2. Results

2.1. Regeneration and identification of the transgenic plants

A total of 500 cell aggregates of Xushu 18 (Fig. 1-A) cocultivated with *Agrobacterium tumefaciens* strain EHA 105 were cultured on the selective medium with 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg L^{-1} carbenicillin (Carb) and 7 mg L^{-1} hygromycin (Hyg). After 8 wk, 52 of them formed Hyg-resistant embryogenic calluses (Fig. 1-B). After transferred to Murashige and Skoog (MS) medium with 1.0 mg L^{-1} abscisic acid (ABA), 100 mg L^{-1} Carb and 7 mg L^{-1} Hyg, 24 of 52 Hyg-resistant calluses produced somatic embryos. These somatic embryos germinated into plantlets on the same medium (Fig. 1-C). A total of 37 putatively transgenic plants, named X1, X2, X3, … , X37, were obtained in this study.

β-glucuronidase (GUS) assay showed that 28 of the 37 putatively transgenic plants had visible GUS activity in leaf, stem and root tissues, which showed stable *gusA* gene integration into the genome of the plants (Fig. 1-D, E, F). PCR analysis indicated that the 28 GUS-positive plants had a specific 1 435 bp band of the *IbMIPS*1 gene, which indicated that they were transgenic (Fig. 1-G). No specific band was observed in the GUS-negative plants and wild-type (WT).

2.2. Improved salt tolerance in the transgenic plants

Twenty-eight transgenic plants exhibited vigorous growth and good rooting in contrast to the poor-growing WT on MS medium with 86 mmol L^{-1} NaCl (Fig. 1-H; Table 1). These results demonstrated that the transgenic plants had the imporved salt tolerance compared to WT.

Proline and malonaldehyde (MDA) contents and superoxide dismutase (SOD) activity of the 28 transgenic plants were analyzed (Table 1). Proline content and SOD activity of the 16 transgenic plants significantly increased, while their MDA content significantly decreased compared to WT, indicating the marked improvement of their salt tolerance.

These 16 transgenic plants and WT were transferred to the soil in a greenhouse and a field, and showed 100% survival. Their cuttings were cultured for 4 wk in the Hoagland solution with 0 and 86 mmol L^{-1} NaCl, respectively. All of cuttings exhibited normal growth and rooting without NaCl. Under 86 mmol L^{-1} NaCl stress, the four transgenic plants (X5, X29, X22, X36) formed new leaves and roots; the six transgenic plants only survived, but failed to form new leaves; the six transgenic plants and WT gradually became brown to death (Fig. 1-I, J; Table 2). Thus, the four transgenic plants, X5, X29, X22, and X36 exhibited significantly enhanced salt tolerance compared to the other transgenic plants and WT.

The four salt-tolerant transgenic plants and WT were grown in a transplanting box and irrigated with a 200-mL of 200 mmol L^{-1} NaCl solution once every 2 d for 4 wk. The salt-tolerant plants exhibited good growth and increased physical size, while WT died (Fig. 1-K and L). Their fresh weight (FW) and dry weight (DW) were increased by 125–314% and 60–201%, respectively, compared to WT (Fig. 1-M).

2.3. Enhanced photosynthesis in the salt-tolerant transgenic plants

Photosynthesis of the salt-tolerant transgenic plants grown in a transplanting box under 200 mmol L^{-1} NaCl stress for

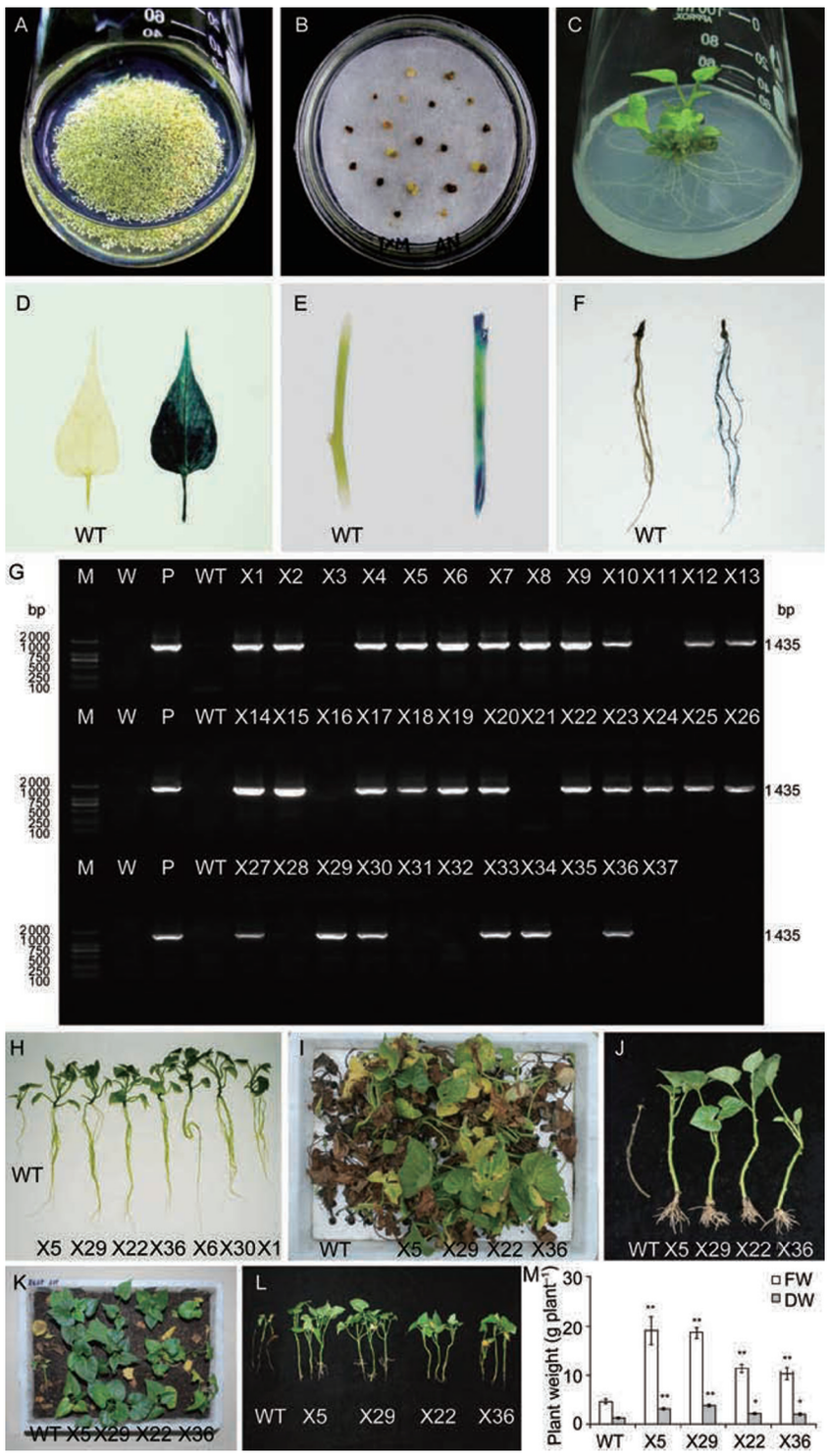

Fig. 1 Production and salt tolerance evaluation of transgenic sweetpotato plants overexpressing the *IbMIPS*1 gene. A, rapidly-proliferating embryogenic suspension cultures. B, Hyg-resistant embryogenic calluses formed on the selective medium after 8 weeks of selection. C, plantlets regenerated from a Hyg-resistant callus. D, E and F, GUS expression in leaf, stem and root of a transgenic plant, respectively. G, PCR analysis of the transgenic plants. M, DL2000 DNA marker; W, water as negative control; P, plasmid pCAMBIA1301-*IbMIPS*1 as positive control; WT, wild-type as negative control; X1, X2, X4–X10, X12–X15, X17–X20, X22–X27, X29, X30, X33, X34, X36, transgenic plants; X3, X11, X16, X21, X28, X31, X32, X35, X37, non-transgenic plants. H, the growth and rooting of X5, X29, X22, X36, X6, X30, X1, and WT on Murashige and Skoog (MS) medium with 86 mmol L^{-1} NaCl. I and J, phenotypes of X5, X29, X22, X36, and WT cultured in Hoagland solution with 86 mmol L^{-1} NaCl. K, L and M, phenotypes, fresh weight (FW) and dry weight (DW) of X5, X29, X22, X36, and WT grown in a transplanting box under 200 mmol L^{-1} NaCl stress. * and **, a significant difference from that of WT at $P<0.05$ and <0.01, respectively, by Student's *t*-test. Values are means±SE. The same as below.

Table 1 Comparison of salt tolerance between the transgenic plants and wild-type (WT) after 4 wk of culture on Murashige and Skoog (MS) medium with 86 mmol L^{-1} NaCl

Plant lines	Proline content (μg g^{-1} FW)[1)]	SOD activity (U g^{-1} FW min^{-1})	MDA content (nmol g^{-1} FW)	No. of roots	Length of roots (cm)
X5	106.73±0.43**	28.74±0.14**	11.76±0.20**	98.33±2.86**	19.67±2.83**
X29	103.43±1.32**	30.63±0.11**	12.06±0.12**	89.33±4.01**	22.37±4.55**
X22	98.98±0.161**	28.31±0.26**	11.01±0.13**	78.67±1.93**	21.57±2.74**
X36	97.18±2.22**	27.55±0.24**	12.26±0.27**	88.33±2.09**	25.80±3.05**
X6	93.04±0.39**	27.84±0.19**	12.19±0.26**	80.00±5.53**	22.23±3.52**
X30	85.07±1.77**	27.62±0.25**	12.21±0.23**	67.33±3.58**	24.40±2.38**
X1	77.43±2.51**	25.78±0.38**	13.24±0.19**	74.67±2.05**	19.87±2.28**
X20	69.15±0.96**	26.51±0.34**	14.10±0.07**	62.33±2.33**	23.37±5.10**
X17	61.87±3.59**	25.40±0.12**	12.94±0.08**	55.00±7.21**	19.60±0.72**
X13	59.31±2.71**	24.53±0.22**	13.33±0.14**	53.33±5.21**	16.97±0.31**
X2	54.83±3.52**	25.76±0.38**	14.32±0.29**	44.33±7.09**	19.67±2.78**
X4	53.91±0.22**	25.00±0.38**	13.42±0.11**	42.33±1.41**	19.17±3.18**
X23	52.92±0.43**	23.19±0.20**	14.10±0.13**	50.00±5.13**	16.80±2.40**
X9	52.80±1.63**	24.15±0.30**	14.48±0.27**	34.00±2.19**	17.93±1.30**
X33	52.73±0.48**	23.84±0.24**	14.51±0.37**	37.33±3.02**	15.60±3.90**
X34	47.12±0.32*	20.81±0.32**	15.47±0.84*	37.00±4.00**	15.37±0.67**
X7	46.51±1.56	23.11±0.23**	14.93±0.29**	28.33±1.26**	12.73±1.36*
X19	46.50±0.83	19.34±0.18	15.45±0.37*	26.33±1.50**	17.96±0.47**
X25	44.42±1.20	18.85±0.27	15.58±0.16*	32.00±3.18**	11.30±1.78*
X15	42.68±1.31	20.12±0.23**	17.71±0.19	25.67±1.24**	19.30±1.78**
WT	39.05±2.26	16.79±0.27	18.27±0.02	7.33±4.71	6.46±2.39
X8	34.96±1.68	17.94±0.21	16.95±0.08*	12.67±3.26*	9.18±2.78
X27	33.89±1.67	9.58±0.26	17.32±0.08*	13.67±4.11*	9.70±2.40
X24	32.47±1.20	11.90±0.33	19.98±0.24	11.33±1.64*	11.40±0.92*
X10	30.27±1.28	12.82±0.16	22.79±0.61	9.67±3.86	9.4 ±2.91
X14	27.63±0.71	14.43±0.08	19.66±0.28	10.67±2.52	9.40±3.25
X12	27.29±0.67	6.88±0.42	23.50±0.61	8.33±4.02	7.50±3.02
X26	25.27±1.00	8.73±0.41	21.31±0.39	4.00±2.10	9.43±4.20
X18	22.23±0.71	8.34±0.30	25.20±1.14	3.67±0.58	4.87±1.22

[1)] FW, fresh weight.
* and **, a significant difference from that of WT at $P<0.05$ and <0.01, respectively, by Student's *t*-test. Values are means±SE. The same as below.

Table 2 Leaf and root formation of the transgenic plants cultured for 4 weeks in Hoagland solution with 86 mmol L^{-1} NaCl

Plant lines	Leaf formation[1)]	No. of roots	Length of roots (cm)
X5	++	26.67±4.33**	7.30±1.01**
X29	++	62.67±6.12**	5.17±0.54**
X22	++	81.33±9.39**	5.57±0.50**
X36	++	24.00±4.04**	5.20±0.15**
X6	+	15.00±3.21**	5.70±0.53**
X30	+	12.67±2.19**	3.27±0.84**
X1	+	10.00±2.65**	2.60±0.25*
X20	+	9.67±2.33*	1.40±0.25
X17	+	7.33±1.86*	1.07±0.32
X13	+	6.67±1.45*	1.13±0.18*
X2	–	4.67±1.20	0.83±0.19
X4	–	5.33±1.45*	0.73±0.24
X23	–	3.00±1.00	0.63±0.09
X9	–	2.33±0.88	0.63±0.07
X33	–	2.67±0.88	0.33±0.13
X34	–	1.33±0.33	0.27±0.03
WT	–	0.67±0.33	0.20±0.12

[1)] ++ indicates that cuttings formed obvious new leaves; + indicates that cuttings survived, but failed to form new leaves; – indicates that cuttings died.

2 wk was measured. The results showed that they had significantly higher photosynthetic rate, stomatal conductance, transpiration rate, and chlorophyll relative content, which were increased by 11–40%, 20–53%, 22–54%, and 20–42%, respectively, compared to WT (Fig. 2).

2.4. Increased inositol content in the salt-tolerant transgenic plants

Inositol content of the salt-tolerant transgenic plants grown in a transplanting box under 200 mmol L^{-1} NaCl stress for 2 wk was measured. They exhibited significantly higher inositol content compared to WT (Fig. 3).

2.5. Reduced H_2O_2 accumulation in the salt-tolerant transgenic plants

Abiotic stress induces the accumulation of H_2O_2, which is the toxic molecule that causes oxidative damage in plants (Apel and Hirt 2004; Liu *et al*. 2014b). H_2O_2 content of the

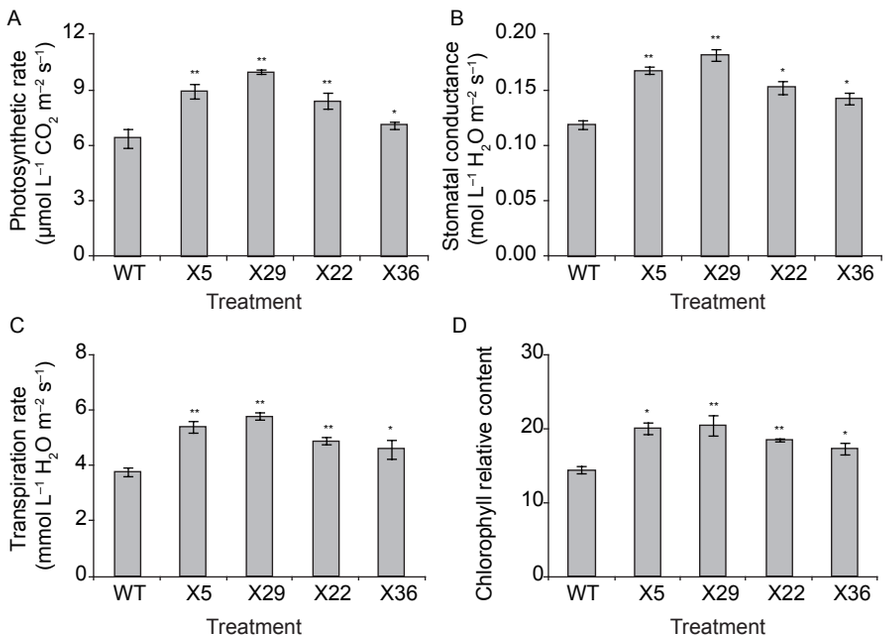

Fig. 2 Photosynthetic performance of the salt-tolerant transgenic plants under salt stress. A, B, C and D, photosynthetic rate, stomatal conductance, transpiration rate, and chlorophyll relative content of X5, X29, X22, X36, and WT, respectively.

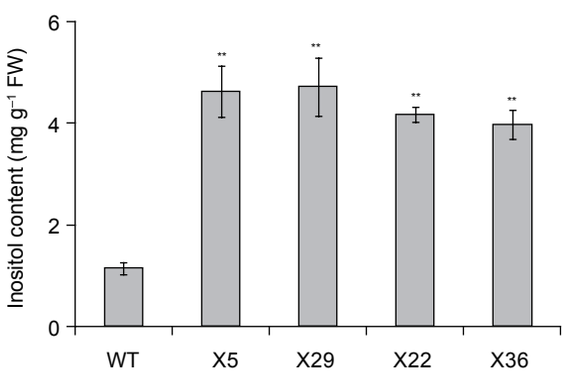

Fig. 3 Inositol content of the salt-tolerant transgenic plants under salt stress.

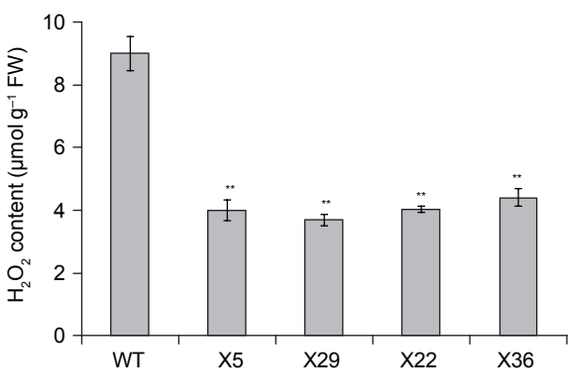

Fig. 4 H_2O_2 content of the salt-tolerant transgenic plants under salt stress.

salt-tolerant transgenic plants grown in a transplanting box under 200 mmol L^{-1} NaCl stress for 2 wk was measured. The results showed that the salt-tolerant transgenic plants had significantly lower H_2O_2 content compared to WT (Fig. 4).

2.6. Southern blot analysis of the salt-tolerant transgenic plants

Transgene integration patterns of the salt-tolerant transgenic plants were analyzed by Southern blot. The results demonstrated they displayed different patterns and the copy number of integrated gene varied from 1 to 2, while WT had no hybridizing band as expected (Fig. 5).

2.7. Expression analysis of the related genes in the salt-tolerant transgenic plants

Expression of inositol and proline biosynthesis and photosynthesis associated genes and *SOD* gene in the salt-tolerant transgenic plants was analyzed by real-time quantitative PCR (qRT-PCR). The results showed that the transgenic plants exhibited significantly higher expression level of *IbMIPS*1 gene compared to WT (Fig. 6). *MIPP* gene and salt stress responsive genes, pyrroline-5-carboxylate synthase (*P5CS*), pyrroline-5-carboxylate reductase (*P5CR*) and *SOD*, were significantly up-regulated in the transgenic plants compared to WT (Fig. 6). The *psbA* gene, encoding D1 protein, and *PRK* gene, encoding phosphoribulokinase

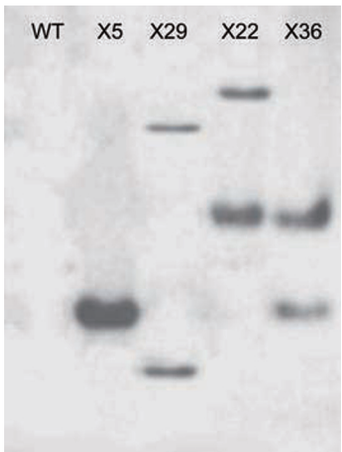

Fig. 5 Southern blot analysis of the salt-tolerant transgenic plants.

(PRKase), were also significantly up-regulated in transgenic plants (Fig. 6).

3. Discussion

Overexpression of salt tolerance associated genes is an alternative strategy for improving salt tolerance of plants. The *MIPS* gene has been shown to enhance salt tolerance in several plant species (Das-Chatterjee *et al*. 2006; Joshi *et al*. 2013; Kaur *et al*. 2013; Tan *et al*. 2013; Goswami *et al*. 2014). This study has indicated that overexpression of *IbMIPS*1 significantly enhanced salt tolerance of sweetpotato (Fig. 1).

MIPS is a key rate limiting enzyme in the *de novo* biosynthesis of inositol in plants. There was a direct correlation between inositol content and salt tolerance in transgenic

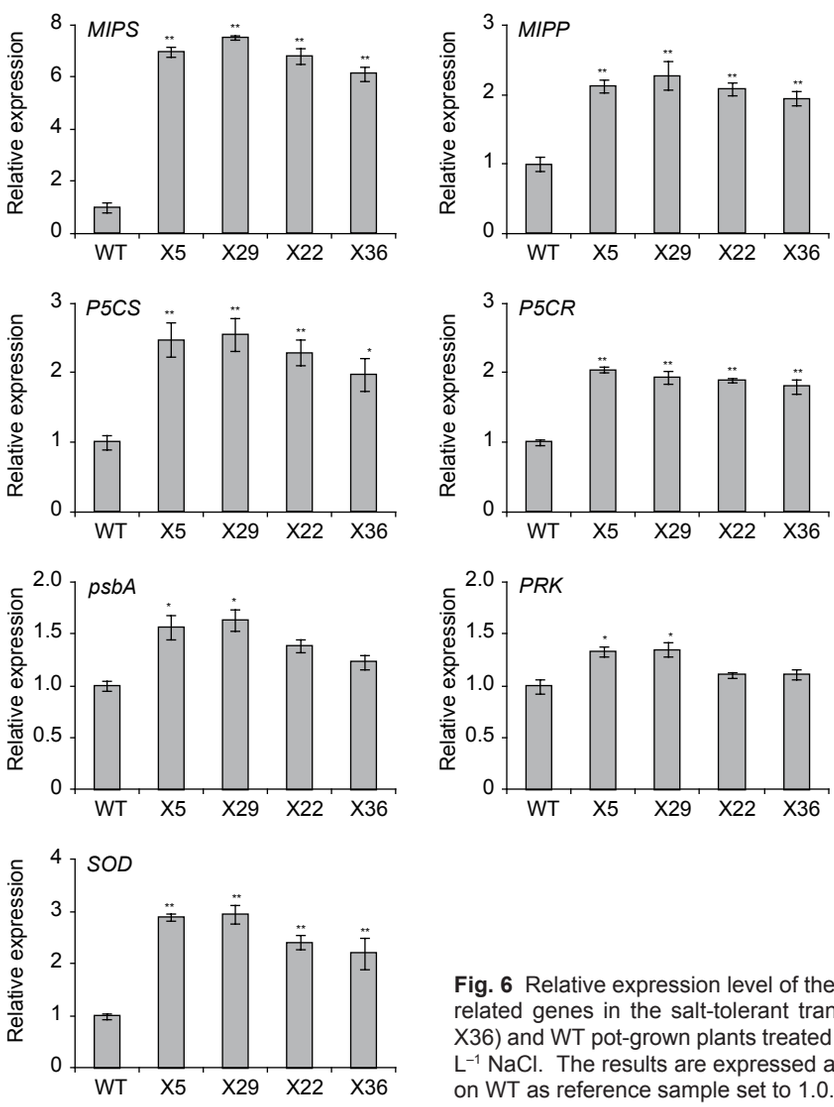

Fig. 6 Relative expression level of the *IbMIPS*1 gene and the related genes in the salt-tolerant transgenic (X5, X29, X22, X36) and WT pot-grown plants treated for 2 wk with 200 mmol L^{-1} NaCl. The results are expressed as relative values based on WT as reference sample set to 1.0.

B. juncea, tobacco and rice plants overexpressing *PcINO*1 (*PcMIPS*1) (Majee *et al*. 2004; Das-Chatterjee *et al*. 2006; Goswami *et al*. 2014). The increase of inositol content enhanced salt tolerance of the *CaMIPS*2-overexpressing *Arabidopsis* (Kaur *et al*. 2013). Overexpression of *MfMIPS*1 increased inositol content, which led to the enhanced tolerance to salt stress in tobacco (Tan *et al*. 2013). In the present study, the *IbMIPS*1-overexpressing sweetpotato plants exhibited significantly higher inositol levels compared to WT under salt stress (Fig. 3). It was also found that the expression levels of *IbMIPS*1 and *MIPP* were significantly higher in the transgenic plants under salt stress (Fig. 6). It is suggested that overexpression of *IbMIPS*1 increases inositol content by up-regulating *MIPP*, which lead to the enhanced salt tolerance of the transgenic sweetpotato plants.

In plants, proline level is related to the extent of salt tolerance (Zsigmond *et al*. 2012; Joshi *et al*. 2014; Liu *et al*. 2014b, c; Surekha *et al*. 2014). In this study, we found that the *IbMIPS*1-overexpressing sweetpotato plants had significantly higher proline content than WT under salt stress, which indicated the marked improvement of their salt tolerance (Table 1). Higher proline level in the *IbMIPS*1-overexpressing sweetpotato plants might protect membrane integrity by maintaining the osmotic balance between the intracellular and extracellular environment, which resulted in the enhanced salt tolerance. The similar results were also reported in several studies (Wyn Jones and Storey 1978; Delauney and Verma 1993; Hare and Cress 1997; De Ronde *et al*. 2004; Zhang *et al*. 2012b; Liu *et al*. 2014b). We also found that *P5CS* and *P5CR*, two key genes in proline glutamate biosynthesis pathway, were up-regulated in the *IbMIPS*1-overexpressing plants under salt stress (Fig. 6). Thus, these results suggest that overexpression of *IbMIPS*1 increases proline level by up-regulating *P5CS* and *P5CR*, which lead to the enhanced salt tolerance of the transgenic sweetpotato plants.

Salt stress induces reactive oxygen species (ROS) production in plant cells. It is important to maintain a stronger ROS-scavenging ability under salt stress to alleviate the induced oxidative damage, especially in plant leaves where photosynthesis is dramatically impacted (Gill and Tuteja 2010). SOD as a key enzyme of ROS scavenging system is usually induced by salt stress to accelerate the dismutation of superoxide into oxygen and H_2O_2, which can be subsequently removed through different pathways (Koca *et al*. 2006; Zhang *et al*. 2012a). Therefore, SOD activity is often used to evaluate the salt tolerance of plants (Mishra *et al*. 2013; Yang *et al*. 2013; Liu *et al*. 2014b, c). In this study, the *IbMIPS*1-overexpressing sweetpotato plants had significantly higher SOD activity and significantly lower H_2O_2 content compared to WT (Table 1; Fig. 4). The expression of *SOD* gene was also increased in the transgenic plants (Fig. 6). Therefore, the improved salt tolerance of the transgenic sweetpotato plants might be due, at least in part, to the enhanced ROS scavenging capacity (Zhang *et al*. 2012a). Similar results were also observed in other reports (Fan *et al*. 2012; Mishra *et al*. 2013; Yang *et al*. 2013; Liu *et al*. 2014b, c). In addition, proline is an effective scavenger of singlet oxygen and hydroxyl radicals (Smirnoff and Cumbes 1989; Alia *et al*. 2001). Higher proline level might increase the expression of *SOD*, which resulted in the enhanced ROS scavenging capacity, in the *IbMIPS*1-overexpressing sweetpotato plants.

In plants, MDA is a marker for lipid peroxidation (Roy-Choudhury *et al*. 2007). Higher MDA content can reduce salt tolerance of plants because it can induce cell membrane damage (Deng *et al*. 2013; Wang *et al*. 2013b; Liu *et al*. 2014b, c). The present study found that MDA content was significantly lower in *IbMIPS*1-overexpressing plants than in WT under salt stress, indicating that the transgenic plants had the enhanced salt tolerance (Table 1).

ROS can inhibit the repair of photodamaged PSII and its primary sites are the oxygen-evolving complex and the D1 proteins (Takahashi and Murata 2008). We found that photosynthetic rate, stomatal conductance, transpiration rate, and chlorophyll relative content were significantly higher in the *IbMIPS*1-overexpressing sweetpotato plants than in WT under salt stress (Fig. 2). qRT-PCR analysis revealed that overexpression of *IbMIPS*1 up-regulated *psbA* and *PRK* (Fig. 6). Thus, it is thought that the biomass difference between the *IbMIPS*1-overexpressing plants and WT is due to the difference of photosynthesis (Fig. 1). The higher level of proline in the transgenic plants could provide protection against photoinhibition, which less affected photosynthesis of the *IbMIPS*1-overexpressing plants under salt stress (Alia *et al*. 1991, 1997; de Ronde *et al*. 2004).

4. Conclusion

Overexpression of *IbMIPS*1 enhanced salt tolerance in transgenic sweetpotato plants. It is thought that the *IbMIPS*1 gene may enhance salt tolerance of sweetpotato by regulating the expression of salt stress responsive genes, increasing the content of inositol and proline and enhancing the activity of photosynthesis. The *IbMIPS*1 gene has the potential to be used to improve salt tolerance in sweetpotato and other plants.

5. Materials and methods

5.1. Plant materials

Sweetpotato cultivar Xushu 18, one of the most important

commercial cultivars in China, was used in this study. Embryogenic suspension cultures of Xushu 18 were established as described by Liu *et al*. (2001). Cell aggregates 0.7–1.3 mm in size were selected from embryogenic suspension cultures for the transformation according to the method of Liu *et al*. (2014b).

5.2. Transformation and plant regeneration

The *A. tumefaciens* strain EHA105 harbors a binary vector, plasmid pCAMBIA1301. This binary vector contains *IbMIPS*1, *gusA* and *hpt*II genes driven by a CaMV 35S promoter, respectively (Fig. 7). The recombinant vector was transformed into EHA 105 for transformation of sweetpotato. The *Agrobacterium* suspension was prepared for the inoculation according to the method of Yu *et al*. (2007). Transformation, selection and regeneration were conducted as described by Liu *et al*. (2014b), but 7 mg L^{-1} Hyg was used for the selection culture.

5.3. GUS assay and PCR analysis

The putatively transgenic sweetpotato plants were tested for GUS expression using histochemical GUS assay according to the method of Jefferson *et al*. (1987). The leaf, stem and root tissues were treated for 12 h in GUS assay buffer at 37°C and the blue staining denoted positive reaction.

Based on the instructions of EasyPure Plant Genomic DNA Kit (Transgen Biotech, Beijing, China), genomic DNA was extracted from the leaves of transgenic plants and WT. Equal amounts of 200 ng of DNA were amplified in 50 mL reactions using specific primers for the *IbMIPS*1 gene (Table 3), which were expected to give products of 1 435 bp. PCR amplifications were conducted with an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, and final extension at 72°C for 5 min. PCR products were separated by electrophoresis on a 1.0% (w/v) agarose gel.

5.4. *In vitro* assay for salt tolerance

To evaluate *in vitro* salt tolerance of the transgenic plants, they were cultured on MS medium with 86 mmol L^{-1} NaCl for 4 wk at (27±1)°C under 13 h of cool-white fluorescent light at 54 μmol L^{-1} m^{-2} s^{-1} as described by He *et al*. (2009). Meanwhile, proline content and SOD activity were measured according to the method of He *et al*. (2009). MDA content was analyzed as described by Gao *et al*. (2011). Three plants were treated for each line.

5.5. *In vivo* assay for salt tolerance

The transgenic plants were transplanted in a greenhouse. To evaluate their *in vivo* salt tolerance, the cuttings about 25 cm in length were cultured for 4 wk in the Hoagland solution (Hoagland and Arnon 1950) containing 0 and 86 mmol L^{-1} NaCl, respectively. After water culture assay, the 25-cm-long cuttings of the transgenic plants were grown in a transplanting box with a mixture of soil, vermiculite and humus (1:1:1, v/v/v) for further evaluation of salt tolerance as described by Liu *et al*. (2014b). Three cuttings were used for each line.

5.6. Analysis of photosynthesis

The salt-tolerant transgenic plants were grown in a transplanting box for 2 wk under 200 mmol L^{-1} NaCl stress and then their photosynthetic rate, stomatal conductance and transpiration rate were analyzed as described by Liu *et al*. (2014b). Relative chlorophyll content (SPAD value in fresh leaves) was measured with the chlorophyll meter SPAD-502 (Minolta, Japan) according to the method of Fernández-Falcón *et al*. (2006). The experiments were performed at 9–11 a.m. of sunny days.

5.7. Measurements of inositol and H_2O_2 content

The salt-tolerant transgenic plants were grown in a transplanting box for 2 wk under 200 mmol L^{-1} NaCl stress and then their inositol and H_2O_2 content were measured according to the methods of Bieleski and Redgwell (1977) and Alexieva *et al*. (2001), respectively.

5.8. Southern blot analysis

Southern blot analysis was conducted according to the

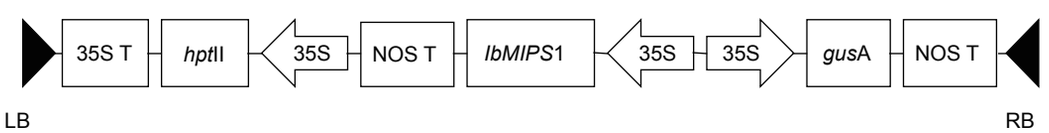

Fig. 7 Schematic diagram of the T-DNA region of binary plasmid pCAMBIA1301-*IbMIPS*1. LB, left border; RB, right border; 35S T, CaMV 35S terminator; *hpt*II, hygromycin phosphotransferase II gene; 35S, cauliflower mosaic virus (CaMV) 35S promoter; NOS T, nopaline synthase terminator; *IbMIPS*1, sweetpotato *myo-inositol*-1-phosphate synthase gene; *gusA*, β-glucuronidase gene.

Table 3 Primers used in the present study

Primer name	Primer sequence (5′→3′)
Primers for PCR	
*IbMIPS*1-PCR-F	ACCTAACAGAACTCGCCGTAAAGAC
*IbMIPS*1-PCR-R	TCCACCAATCAAACTGTTCCTCCTG
Primers for Southern blotting	
hpt II-F	ACAGCGTCTCCGACCTGATGCA
hpt II-R	AGTCAATGACCGCTGTTATGCG
Primers for qRT-PCR	
Actin-F	AGCAGCATGAAGATTAAGGTTGTAGCAC
Actin-R	TGGAAAATTAGAAGCACTTCCTGTGAAC
MIPP-F	ACAAGTTCAGGCTGGGAAGACA
MIPP-R	ACTGACAAGGCATGTGAAGACC
*MIPS*1-F	AGGAGGAACAGTTTGATTGGTGGAG
*MIPS*1-R	CGGAAAGTTTGAGGGGCAGATAGGT
P5CR-F	ATAGAGGCATTGGCTGATGG
P5CR-R	GGTAGTCCCACCTGGTGATG
P5CS-F	GCCTGATGCACTTGTTCAGA
P5CS-R	TTGAGCAATTCAGGGACCTC
PRK-F	GCTCTCAACATAGATCAGCT
PRK-R	TGAAGGCTCTACTATCTCAT
psbA-F	CATCCGTTGATGAATGGTTA
psbA-R	GCAACAGGAGCTGAGTATGC
SOD-F	TCCTGGACCTCATGGATTTC
SOD-R	GCCACTATGTTTCCCAGGTC

method of Liu *et al*. (2014c). Coding sequence of the 591 bp *hpt*II was used as probe (Table 3). The labeling of probe, prehybridization, hybridization and detection were conducted using DIG High Prime DNA Labeling and Detection Starter KitII (Roche, Basel, Switzerland).

5.9. Expression analysis of the related genes

The salt-tolerant transgenic plants were grown in a transplanting box for 2 wk under 200 mmol L^{-1} NaCl stress and then the expression of inositol and proline biosynthesis, photosynthesis and *SOD* genes was analyzed by qRT-PCR according to the method of Liu *et al*. (2014b). Specific primers were designed from conserved regions of genes (Table 3). Sweetpotato *β-actin* gene was used as an internal control (Table 3). Quantification of the gene expression was conducted with comparative C_T method (Schmittgen and Livak 2008).

5.10. Statistical analysis

All of the experiments were repeated three times. The data were presented as the mean±SE and analyzed by Student's *t*-test in a two-tailed analysis to compare the parameters obtained under normal and salt stress conditions. A *P*-value of <0.05 or <0.01 was considered to be statistically significant. * and ** indicated a significant difference from that of WT at *P*<0.05 and <0.01, respectively.

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