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EXPRESSION OF *PORPHYRA YEZOENSIS TPS* GENE IN TRANSGENIC RICE ENHANCED THE SALT TOLENRANCE

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A B S T R A C T

The trehalose-6-phosphate synthase gene of Porphyra yezoensis (*PyTPS*) was isolated and cloned into a plant gene expression vector pCAMBIA2300-35S-*OCS*, and the resulting construct pCAMBIA2300-*PyTPS* was transformed into *Agrobacterium tumefaciens* (*A. tumefaciens*) strain AGL1. Genetic transformation of rice variety TP309 was performed with the *A. tumefaciens* containing pCAMBIA2300-*PyTPS*. After antibiotic G418 screening and PCR analysis, one hundred T_0 transgenic plants were seclected and transplanted into the trial field in the greenhouse and used for further study. Ninety-five of these 100 T_0 transgenic cultivaries produced their seeds, which were harvested and stored separately. All of the 95 potential T_1 transgenic lines were re-identified by PCR analysis, and their salt-tolerance was tested with 3‰ and 5‰ NaCl solutions. Results indicated that 78 of the 95 T_1 transgenic lines were PCR- positive and resistant to 5‰ NaCl solution. Salt-tolerance of these 78 T_1 transgenic lines was further tested with higher concentration of NaCl solutions. Of which, three lines (H155, H191 and Y308) showed resistance to 8‰ NaCl in the test. These 3 lines were comprehensively analyzed by PCR, Southern hybridization, northern hybridization and RT-PCR analyses. In addition, trehalose content measurement and preliminary yield evaluation were carried out, results indicated that the *PyTPS* gene was integrated into the genomic DNA sequences of these 3 transgenic lines and expressed indeed in the transgenic plants. Detection of the transformed *PyTPS* gene in these 3 transgenic lines was performed in plants from T_1 to T_6 generations; results indicated that the transformed *PyTPS* gene was present in transgenic plants from T_1 to T_6 generations.

Keywords: *Agrobacterium*-mediated transformation, *Porphyra yezoensis* Salt-tolerance, Transgenic rice, Trehalose-6-phosphate synthase (TPS).

INTRODUCTION

In the world where human population growth is outstripping the grain supply, new agricultural techniques, especially plant biotechnology such as gene transformation, should be swiftly implemented to narrow the gap between grain production and human need (Altman and Hasegawa, 2012). Soil salinity is one of the most important stresses for crop production. Introducing exotic salt-resistance gene by genetic transformation is a commonly used method for crop salt-resistance breeding. Trehalose (α-D-glucopyranosyl-(1, 1)-α-D-glucopyranoside) is a non-reducing disaccharide of two glucose units presented throughout the bacterial, fungal, yeast, animal and plant kingdoms (Elbein, 1974; Vuorio *et al.,* 1993; Kaasen *et al.,* 1994; Goddijn and Smeekens, 1998; Lowe *et al.,* 2009), and often functions as a stress protection metabolite in the stabilization of biological structures under stress tolerance (Goddijn and van Dun, 1999; Eastmond *et al.,* 2002). Trehalose accumulates much higher amount in bacteria and yeast than in other organisms (Elbein, 1974), but it is present only in trace amount in crop plants, which is not adequate for a role in stress protection (Muller *et al.,* 1999; Vogel, 2001; Lunn *et al.,* 2006). In many organisms, biosynthesis of

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trehalose is a two-step process, which consisted of the conversion of UDP-glucose and glucose-6-phosphate into trehalose-6-phosphate (T6P) by trehalose-6-phosphate synthase (TPS), and subsequent dephosphorylation of the T6P by trehalose-6-phosphate phosphatase (TPP) (Eastmond *et al.,* 2002; Paul *et al.,* 2008). In bacteria and yeast, TPS and TPP are two separate domains encoded by *TPS* and *TPP* gene, respectively.

In the past two decades, many efforts were made to increase the salt-tolerance of plants by foreign *TPS* gene transformation. Some data showed that *TPS* genes from bacteria and yeast were expressed in transgenic plants, and it indeed enhanced the salt-tolerance of transgenic plants (Holmstrom *et al.,* 1996; Pilon-Smits *et al.,* 1998; Garg *et al.,* 2002; Jeong *et al.,.* 2007; Mu *et al.,* 2007; Jia *et al.,* 2007). Considering the fact that seaweeds live in sea water during their whole life history, it is believed that it should be possible to isolate high salt-tolerance genes from the seaweed, and the seaweed *TPS* genes may have potential usage in developing salt-tolerant crop varieties by genetic transformation. Recently, Zhao *et al.* (2013) reported the successfully development of salt-tolerant rice lines by transforming a *TPS* gene (*ZmTPS*) cloned from a sea water grass (the eelgrass *Zostera marina*) into rice variety ZH11, and proved the availability to express sea water *TPS* gene in the land crops. This paper will report the expression of a cloned seaweed *TPS* gene (*PyTPS*) in rice variety TP309 enhanced the salt-tolerance of the transgenic plants. The objective of this study is to search an applicable way for crop salt-tolerance breeding.

MATERIALS AND METHODS

Gene, bacterial strain and rice variety: The *PyTPS* gene was cloned in our laboratory, its accession number in GenBank is AY729671 (Wang *et al.,* 2010). The *A. tumefaciens* strain AGL1 and the rice variety TP309 (*Oryza sativa* L.) are our own laboratory storages.

DNA and RNA extraction: DNAs were extracted from young leaves of rice plants as described by Wang *et al.,* (2003), and used in PCR and Southern hybridization analyses. The total RNA was extracted by guanidinium thiocyanate (GT) method as reported (Chomczynski and Sacchi, 1987) and used for cDNA synthesis. The synthesized cDNA was used in RT-PCR and northern analyses.

Construction of plant gene expression vector pCAMBIA2300-*PyTPS*: The coding sequence of *PyTPS* gene had been cloned into pMD18-T from the *Nde*I and *Hin*dIII sites in our previous work (Wang *et al.,* 2010). In the beginning of this study, the *PyTPS* gene was re-isolated from the recombinant plasmid pMD18-T-*PyTPS* by *Sal*I and *Xba*I double digestion, and then was sub-cloned into a plant gene expression vector pCAMBIA2300-35S-OCS through the corresponding restriction sites.

Transformation of rice variety TP309 with the *PyTPS* gene: Rice variety TP309 was transformed with *Agrobacterium* strain AGL1/pCAMBIA2300-*PyTPS* following the reported method (Hiei *et al.,* 1994) with minor adjustments. The brief procedure is as follows: Rice calli induced from TP309 mature embryos were transformed by *Agrobacterium* strain AGL1/pCAMBIA2300-*PyTPS.* In the first round screening, the transformants were selected based on their G418-resistance in the G418 (50 mg/L) containing selective medium. Then the G418-resistant calli were transferred to the regeneration medium and cultured under the photoperiod of 15 h illumination. The regenerated plants about 2 cm height were transferred into the rooting medium. When the plants reached 10 cm height, the tube lids were pulled out. After 2 days cultivation in culture room, the rice plants were transferred into soil in greenhouse. When they grew normally, DNAs were isolated from their leaves. The second round screening of the potential transgenic plants was performed by PCR analysis. The screened PCR positive plants were considered as T_0 transgenic plants.

Salt-tolerance test of transgenic rice plants: Preliminary salt-tolerance test of young seedlings was performed with 5‰ and 8‰ NaCl solutions respectively on the 95 T_1 transgenic lines following the hydroponic method reported by Zhao *et al.* (2013). Briefly as follows: rice seeds were growing in holes of the floating plastic board placed on water surface. The bottom of holes on the plastic board was sealed with nylon net. In each hole, more than 10 individual seeds of one rice line were planted. The seeds germinated and grew in water for 10 days. After that, the water was moved, and NaCl solution was added instead of water. The salt treatment was last for 10 days. Plant growing was observed frequently during the 10 days salt treatment period. The screened lines with the capacity resistant to 5‰ NaCl solution were selected and then subjected to higher and wider range salt gradient solutions (3%, 5%, 8%, 10%, 12%) in culture bottles.

Molecular characterization of the *PyTPS* transgenic rice - PCR identification: In all of the PCR amplifications, DNAs were extracted from leaves of young plants, and the PCR reactions were performed in MJ-100 PCR machine (USA). All primers used in this study were shown in Table 1. Primer-pair P1/P2 was used in all of the PCR identifications. For T_1 transgenic plants, the PCR identification was performed with all of the 3 primer-pairs given in Table 1. The total volume of PCR reaction mixture was 25 μL containing 2.5 μL 10× PCR buffer, 1 μL of template DNA (30 ng/μL), 1 μL of each primer set (10 pmol/μL), 1 μL of $MgCl_2$ (50 mmol/L), 1 μL of dNTP (10 mmol/L), and 1 unit of *Taq* DNA polymerase. The reaction was performed at 94℃ for 4 min, and then subjected to 35 cycles of 94℃ for 1 min, 59℃for 1 min, and 72℃ for 1 min, plus a final extension at 72℃for 10 min. The PCR products were then separated on 1%agarose gel and visualized by ethidium bromide (EB) staining.

Table 1. Primers used for PCR identification of transgenic rice plants and their amplification products.

Primer*	Sequence (5′ to 3′)	Product size
P1 (F)	CTACGCGCGTCACTTTCTCTC′	
P2 (R)	CATGATGCTGTACAGCGCAAG	~500 bp
P3 (F)	AGGGAGGACGCACAATCCCACTAT	
P4 (R)	GCACGCAAGCGGAGAGAAAGTGACG	~1050 bp
NPT Ⅱ (F)	TCCGGTGCCCTGAATGAACT	
NPT Ⅱ (R)	GGCGATACCGTAAAGCACGA	581 bp

* F: Forward; R: Reverse

Southern and northern hybridizations: Southern and northern hybridization analyses of rice plants were performed as described by Sambrook *et al.* (1989). In Southern hybridization, the plant genomic DNA was digested with restriction enzyme *Eco*RI (screened from 4 examined restriction enzymes in pre-experiments) and then transferred onto Hybond-N^+ membrane. The membrane was hybridized with a ^{32}p-labeled 500 bp PCR fragment amplified with primer-pair P1/P2 (Table 1). In northern hybridization, cDNA was synthesized on the total RNA isolated from young leaves of rice plants and then hybridized with the same probe as in Southern hybridization.

RT-PCR analysis: Total RNA isolated from young leaves of rice plants was used as template to synthesize the cDNA with the cDNA synthesis kit (Promega, USA) according to the guidance provided by the Company. Primers used in reverse transcription-polymerase chain reaction (RT-PCR) analysis were shown in Table 2. The total volume of PCR reaction mixture was 25 μL containing 2.5 μL 10× PCR buffer, 1 μL of template DNA (30 ng/μL), 1 μL of each primer set (10 pmol/μL), 1 μL of $MgCl_2$ (50 mmol/L), 1 μL of dNTP (10 mmol/L), and 1 unit of *Taq* DNA polymerase. The reaction was performed at 94℃ for 4 min, and then subjected to 30 cycles of 94℃ 1 min, 58℃ 1 min, and 72℃ 1 min, plus a final extension at 72℃ for 10 min. A 446 bp fragment of rice *actin1* gene was amplified and used as a standard internal control in the experiment. The PCR products were separated on 1.0% agarose gel. All of the tests were repeated for 3 times. Table 2. Primers for RT-PCR analysis of T_2 transgenic plants and their amplification products.

Primer*	Sequence (5′ to 3′)	Product size
NGSP2 (F)	GACCTGTCATGGATGGAGT TGGCATTG	
TPS4 (R)	AGCCAGAAAGGGGGAGG AAAG	~700bp
OsActin1-F	GAGGCGCAGTCCAAGAGGG GTAT	446bp
OsActin1-R	TCGGCCGTTGTGGTGAATG AGTAA	

* F: forward; R: reverse

Relative electrical conductivity determination: Relative electrical conductivity (REC) of young leaves was determined according to the reported method (Sukumaran *et al.,* 1972) with minor modifications. The main procedure is as follows: Three young leaves were collected from each rice line (as 3 repeats), washed with distilled water for 3 times. Water was absorbed with filter paper. From each leaf, one gram of tissue was taken and put into a 50 mL flask, put in 4℃ overnight. Next morning, 30 mL ion-free water was added into each flask, the flaks were put at room-temperature for 15 h. Then electrical conductivity (EC1) was measured with an electrical conductivity detector (Shanghai Kangyi, Model DDS-320). The flasks were set in boiling water for 15 min. After cooling down to room-temperature, electrical conductivity (EC2) was measured again. The REC was calculated as follows: REC=EC1/EC2 X 100%.

Trehalose extraction and quantification: Trehalose was extracted and quantified as described by Lunn *et al.* (2006) and briefly as follows: The 3 T_2 transgenic lines (H155, H191 and Y308) and the non-

transformed control line TP309 were assayed. Leaves from the three-week-old young plants were ground into fine powder in liquid nitrogen with a pre-cold mortar. Each treated sample (0.1 g fresh weight) was dispersed in 1 mL of chloroform/acetonitrile (3:7, v/v) and shaken at -10°C for 2 h. Trehalose was extracted 2 times from the organic phase with 200 µL of water at 4°C by vigorous shaking for 5 min, and then centrifuged at 12,000×g for 5 min. The aqueous supernatants were collected and lyophilized. The dried samples were dissolved in 1 mL of double-distilled water and filtered with a 0.45 µm Whatman filter. Quantitative analysis of trehalose was carried out by high-performance ion chromatography with a Sugar-pak-1 column (0.65 cm × 30 cm) using the Waters 244-type HPLC system. All of the experiments were repeated for 3 times. The final provided content results were based on the 3 independent experiments.

Yield evaluation: The transgenic lines have not got permission to release, therefore the yield evaluation experiments were carried out in the controlled greenhouse. Seeds of the T_6 generation were planted in large-size flower pots filled with soil. When the seedlings reached the height about 8 cm, five seedlings were selected and kept in each pot and the others were discard. Later, when they reached the height about 15 cm, salt-stress started by changing fresh-water instead with 8‰ NaCl solution. After that, fresh-water and 8‰ NaCl solution were alternatively changed once a week. Liquid level was labeled on the pot-wall. Salt-stress was stopped at late heading stage and back to normal management till the harvest.

RESULTS

Construction of plant gene expression vector pCAMBIA2300-*PyTPS:* The expression vector generated as described in "Materials and methods" section was named pCAMBIA2300-35S-OCS-*PyTPS* and abbreviated as pCAMBIA2300-*PyTPS* (Fig. 1), in which the *PyTPS* gene was under the control of CaMV 35S promoter. The regenerated expression vector pCAMBIA2300-*PyTPS* was then introduced into *Agrobacterium* strain AGL1, and the generated recombinant strain AGL1/pCAMBIA2300-*PyTPS* was used to transform rice.

Generation of transgenic rice plants: For functional verification, the *PyTPS* gene was transformed into the rice variety TP309 by the *Agrobacterium*-mediated method. In the first screening cycle, the transformants were screened via antibiotic G418-resistance. As a result, a total of 793 potential transgenic plants (T_0) were selected and then transferred into soil in a greenhouse. When the plants reached about 12 cm height, the second srcreening cycle was performed by PCR amplification with primer-pair P1/P2 (Table 1), and results indicated that 625 of the potential 793 T_0 transgenic plants showed PCR positive. After four weeks cultivation in greenhouse, one hundred strong and healthy T_0 transgenic plants were selected and grown untill final seed-harvest. Ninety-five of them produced their T_1 seeds, which were harvested and stored separately and carefully. The T_1 seeds were sown in field in the greenhouse and 95 T_1 lines were developed. Then the young T_1 transgenic plants were identified by PCR amplification with all of the 3 primer-pairs shown in Table 1. Results exhibited that all of the 95 T_1 lines were PCR positive, while the non-transformed TP309 was negative in all cases. Fig. 2 showed part testing result of the T_1 transgenic lines.

Salt-tolerance test of the T_1 transgenic rice plants: Salt-tolerance tests in 5‰ and 8‰ NaCl solutions were carried out on all of the 95 T_1 transgenic lines. Seventy-eight of the 95 T_1 transgenic lines showed resistance to 5‰ NaCl solution (Fig. 3a). Salt-tolerance of these 78 T_1 transgenic lines was further tested with a wide range of salt gradient NaCl solutions (3‰, 5‰, 8‰, 10‰, 12‰). Of these 78 lines, three lines (H155, H191 and Y308) showed resistance to 8‰ NaCl solution. As an example, the testing results of H191, one of the 3 lines, were shown in Fig. 3b. The seeds (T_2) of these 3 lines were harvested and kept separately and used for further study.

Molecular characterization of the obtained 3 high salt-tolerance T_2 transgenic lines: The 3 high salt-tolerance T_2 transgenic rice lines (H155, H191 and Y308) exhibiting resistance to 8‰ NaCl solution were then subjected to further molecular identification including PCR, Southern hybridization, northern hybridization and RT-PCR analyses. DNAs extracted from young leaves of 3 individual plants of each rice line were amplified with primer-pair P1/P2 (Table 1). The expected PCR product was observed in every T_2 transgenic plant, but not in the non-transformed control plant TP309 (Fig. 4a). Southern hybridization results were shown in Fig. 4b, and results indicated that the 3 transgenic lines were positive, while the controls (TP309) were negative. However, the numbers of hybridization band shown in the 3 transgenic lines were different: H155 exhibited 2 hybridization bands, H191 and Y308 exhibited only one band. Northern hybridization results were shown in Fig.

4c. All of the 3 transgenic lines exhibited positive results with only one hybridization band, and the control plant TP309 had no hybridization signal. RT-PCR results indicated that all of the 3 T_2 transgenic lines exhibited positive results following treatment with water or salt solutions, the signal intensities in the salt treatments were much stronger than that of water treatment; but the control plant TP309 didn't produce any amplification product when treated with water or with salt solution (Fig. 4d).

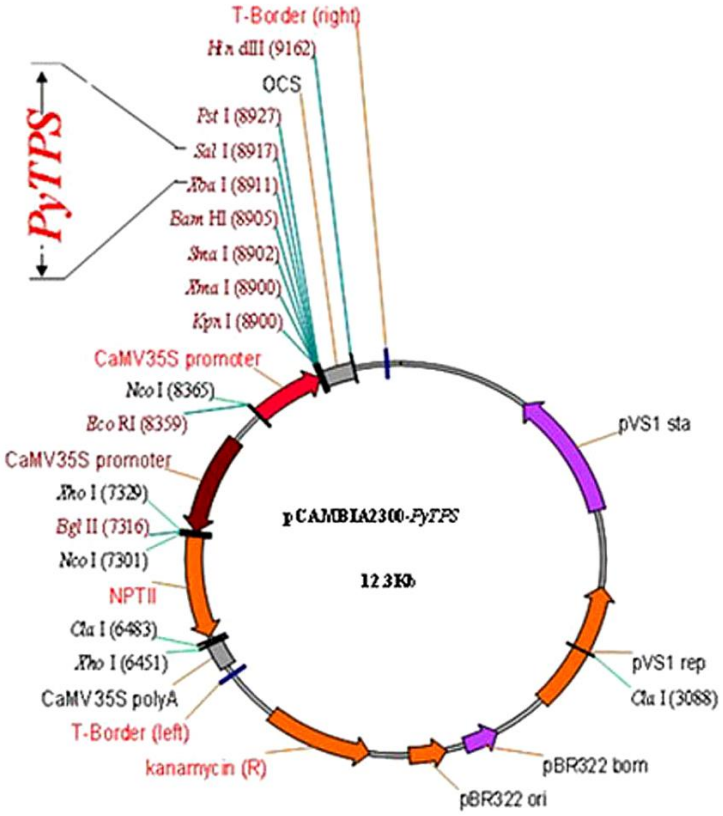


Figure 1. Structure map of the plant expression vector pCAMBIA2300-*PyTPS*.

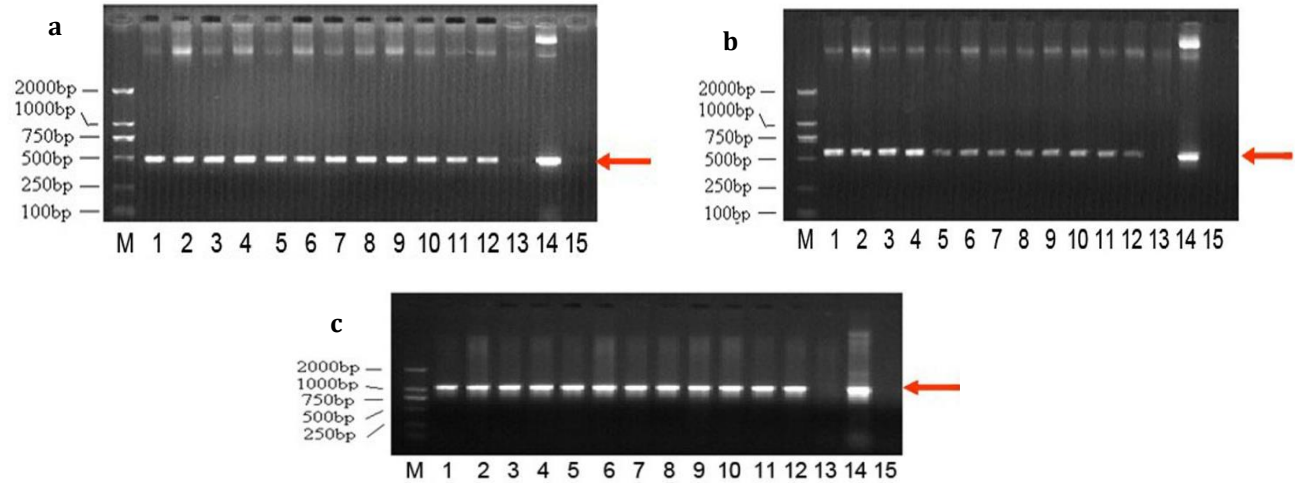


Figure 2. PCR identification of the T_1 transgenic plants. (a), (b) and (c) show the PCR patterns amplified with primer-pairs P1/P2, NPT Ⅱ-F/NPT Ⅱ-R and P3/P4 respectively. M: DNA marker (DL2000); Lanes 1-12: T_1 transgenic plants; Lane 13: non-transformed plant (negative control 1); Lane 14: pCAMBIA2300-*PyTPS* (positive control); Lane 15: water (negative control 2). Arrows indicate the specific PCR products

Genetic transmission of the *PyTPS* gene in transgenic rice plants: So far, the 3 high salt-tolerant transgenic rice lines exhibited stable in the T_6 generation. A genetic transmission test (by PCR amplification) was performed from T_0 to T_6 generations of these 3 high salt-tolerant transgenic lines with the primer-pair P1/P2. In each generation, ten individuals of each selected line were assayed. The detected PCR-negative individuals were replaced with PCR-positive ones of the same line in the test of next generation. Results indicated that the *PyTPS* gene was absent in a few individuals in T_0 and T_1 generations and absent only one in the T_2 generation in the 10 assayed individuals and present in all of the transgenic plants from the T_3 to the T_6 generations. As an example, Fig. 5 showed the detected transmission result in the transgenic line H191.

REC determination: REC determination results were shown in Fig. 6. In normal water conditions no remarkable change was observed in all plants. However, after treatment with NaCl solutions, RECs of all plants increased, but the levels of increases in transgenic plants were obviously lower than those of the control groups.

Measurement of trehalose content: TPS is the key enzyme to catalyze the first and the most important step in the trehalose biosynthesis pathway. Therefore, in this experiment obtaining the increased trehalose content in transgenic plants will be a strong evidence to prove the *PyTPS* gene was successfully transformed into and expressed in receptor plants. The results of Fig. 7 indicated that the transgenic plants exhibited higher trehalose content comparing with the non-transformed control plants both in normal and salt-stressed conditions (treated with 8‰ NaCl solution). In normal conditions, a very low but detectable amount of trehalose was measured in the non-transformed control plants (14.4 ± 1.1 μg•g $^{-1}$ FW).While the trehalose contents in the 3 transgenic lines were 42.6 ± 1.7, 45.8 ± 1.8 and 43.5 ± 1.6 μg•g $^{-1}$ FW, respectively. The trehalose contents in the 3 transgenic lines were 2.95-3.18 times as high as that in non-transformed control plants. In salt-stressed conditions, the trehalose contents both in the transgenic lines and in the non-transformed control plants increased about 70% comparing with that in normal conditions.

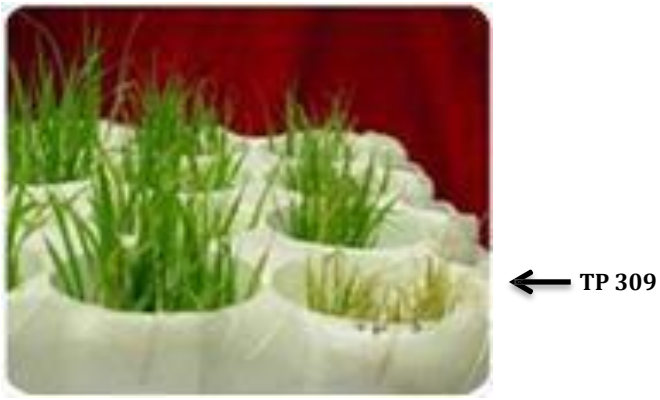
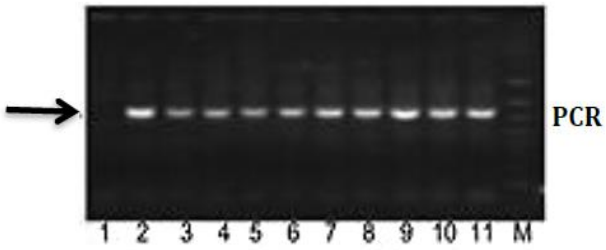
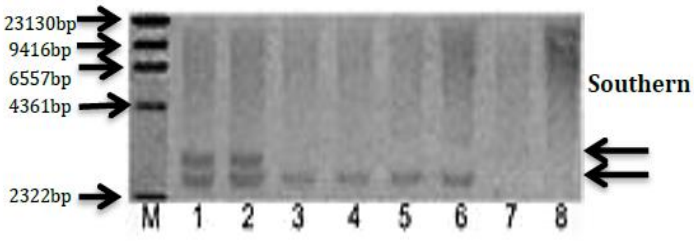


Figure 3(a). T_1 transgenic rice plants treated with 5‰ NaCl solution. Arrow indicates the non-transformed control rice variety TP309, others are T_1 transgenic rice plants. In every hole, there were more than 10 individual plants of the same one T_1 transgenic line

Figure 3(b). T_2 transgenic line H191 treated with wide range NaCl solutions. The concentrations of the NaCl solutions were as follows: 1: 0 (water); 2: 3‰; 3: 5‰; 4: 8‰; 5: 10‰; 6: 12‰. Non-transformed control (TP309) please refers to Fig. 3a





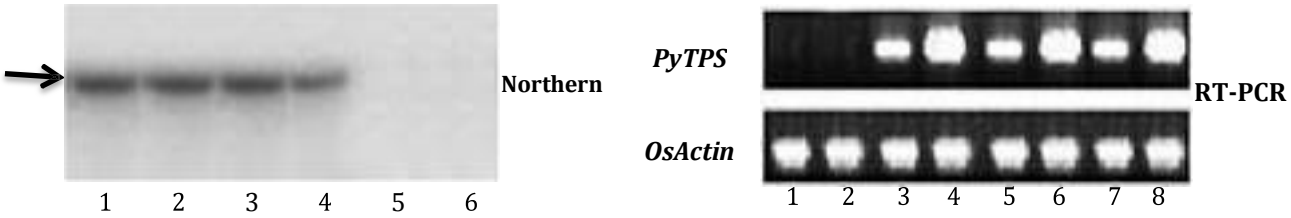


Figure 4. Molecular characterization of the T_2 transgenic lines. (a) PCR identification. Lane 1: non-transformed rice variety TP309 (-ve control); Lane 2: pCAMBIA2300-PyTPS (+ve control); Lanes 3-5: T_2 transgenic line H155; Lanes 6-8: T_2 transgenic line H191; Lanes 9-11 T_2 transgenic line Y308; M: DNA marker (DL2000). Arrow indicates the specific PCR product; (b) Southern hybridization analysis. M: DNA marker (λDNA/*Hin*dIII); Lanes 1 and 2: T_2 transgenic line H155; Lanes 3 and 4: T_2 transgenic line H191; Lanes 5 and 6: T_2 transgenic line Y308 ; Lanes 7 and 8: non-transformed rice variety TP309 (-ve control). Arrows indicate the hybridization bands; (c) Northern hybridization analysis. Lanes 1 and 2: T_2 transgenic line H155; Lane 3 and 4: T_2 transgenic line H191; Lanes 5 and 6: T_2 transgenic line Y308; Lanes 7 and 8: non-transformed rice variety TP309 (-ve control). Arrows indicate the hybridization bands; (d) RT-PCR analysis. Lanes 1 and 2: non-transformed TP309; Lanes 3 and 4: T_2 transgenic line H155; Lanes 5 and 6: T_2 transgenic line H191; Lanes 7 and 8: T_2 transgenic line Y308. Lanes 1, 3, 5, 7: treated with water for 24 h; Lanes 2, 4, 6, 8: treated with 8‰ NaCl solution for 24 h.

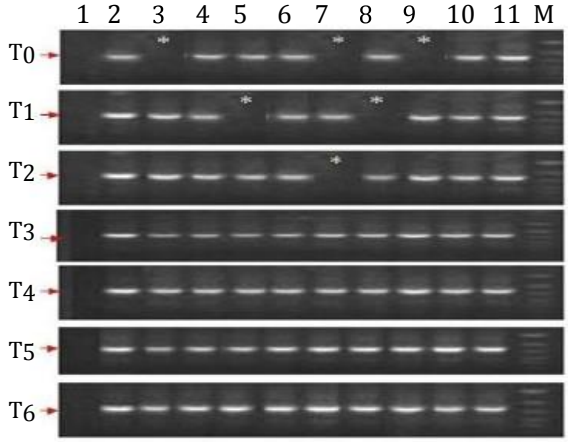


Figure 5. Genetic transmission of the *PyTPS* gene in the progeny of the transgenic rice line H191. Primer-pair P1/P2 was used in the PCR amplification. Lane 1: non-transformed plant; Lanes 2–11: the randomly selected 10 transgenic plants from each generation of the transgenic line H191; M: DNA marker (DL2000). Arrows indicate the specific PCR products. * shows the individual exhibited PCR-negative result. In every generation ten individual plants of each selected line were assayed. The detected PCR-negative individuals were replaced with PCR-positive ones of the same line in the test of next generation.

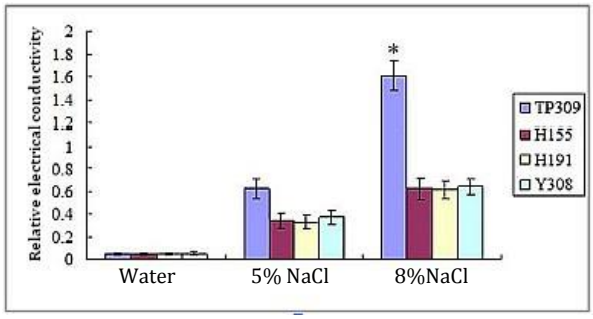


Figure 6. REC changes of rice plants with and without salt stress treatment. Salt stress treatment was performed in different NaCl solutions for 24 h. One gram of three-week old leaf tissue was used as material for REC determination. All given data were based on 3 repeated experiments.

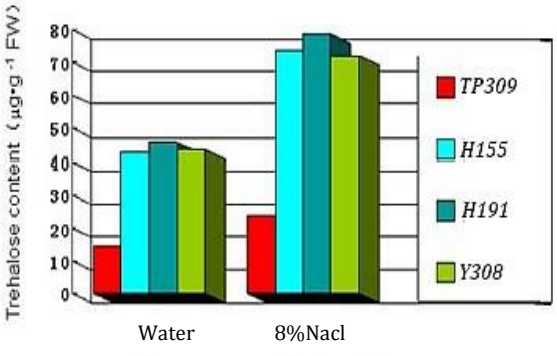


Figure 7. Measurement of trehalose content. For salt stress, the seedlings were growing in water for 2 weeks. After that, they were growing in 8‰ NaCl solution instead of water for 24 h. Then leaves were collected and used for measurement of trehalose content.

YIELD EVALUATION

The preliminary yield evaluation results shown in Table 3 indicated that in salt-stress conditions the growth of non-transformed control plants was inhibited to some extents; and the harvested seed numbers and the thousand-seed weight (TSW) were decreased; the total seed weight is only 36.33% of normal conditions. On the contrary, all of the 3 high salt-tolerance transgenic lines (T_6 generation) only showed slight decline in total seed-numbers and the total weight; their TSW was nearly the same or a little bit higher than that of the non-transformed control plants.

Table 3. Harvested seed-numbers of rice lines in salt-stress and normal conditions.

Rice lines	Salt stress conditions		Normal conditions		Stress/Normal (%)		
	Total seed-numbers	TSW (g)	Total seed-numbers	TSW (g)	Total seed-numbers	TSW	Total weight
TP309	7240±56	25.4±0.5	18540±45	27.3±0.2	39.01	93.04	36.33
H155	17992±45	27.4±0.3	18280±61	27.3±0.3	96.83	100.37	98.60
H191	18016±63	27.5±0.3	18478±52	27.3±0.2	97.50	100.73	98.21
Y308	18351±81	27.3±0.4	18653±59	27.3±0.2	98.40	100.00	98.38

DISCUSSION

***PyTPS* gene and rice transformation:** *PyTPS* gene was transformed into rice variety TP309 via *Agrobacterium*-mediated method. After cultivation in the greenhouse, molecular identification, and salt-tolerance tests, three transgenic lines tolerant to 8‰ NaCl solution were selected. PCR and Southern hybridization results indicated that the *PyTPS* gene was integrated into the genomic DNA of the transgenic rice lines. Transcript expression analyses, (northern hybridization and RT-PCR analysis), salt-tolerance assays, REC and trehalose content measurement results together suggested that the expression of the transformed *PyTPS* gene improved the salt-tolerance of the transgenic rice plants. Trehalose plays an important role in plant cells protecting under stress conditions. In recent years, many papers reported the transformation of *TPS* and *TPP* genes from bacteria and yeast into higher plants (Holmstrom *et al.,* 1996; Pilon-Smits *et al.,* 1998; Garg *et al.,* 2002; Jeong *et al.,* 2007; Mu *et al.,* 2007; Jia *et al.,* 2007). Zhao *et al.* (2013) mentioned that there were some advantages to transform crops by using *TPS* genes from sea water plant instead of the *TPS* and *TPP* genes from bacteria and yeast, and firstly transformed a *TPS* gene (*ZmTPS*) from a sea water grass into rice variety ZH11. This paper reports a similar result regarding the development of salt-tolerant rice lines by transforming a seaweed *TPS* gene (*PyTPS*) into rice variety TP309. *PyTPS* is one of the 10 seaweed *TPS* genes cloned by our laboratory recently (Wang *et al.,* 2010), and the transformations of other cloned seaweed *TPS* genes into rice are undergoing in our laboratory. It is expected to get more available *TPS* genes for rice transformation and salt-tolerant breeding.

The generation of high salt-tolerant transgenic rice lines and the stable inheritance of the transformed *PyTPS* gene in the transgenic rice plants provide another kind of evidences to support the idea that the seaweed *TPS* gene transformation has practical applications in crop salt-tolerance breeding. We believe that in the near future, more and more seaweed *TPS* genes will be transformed into crop plants, which will potentially make great contributions to increase the grain yields to meet the increasing requirements of the rapid growing human population.

Nevertheless, it must be realized that various physiological and biochemical parameters affect the salt-resistance of plant, such as carbon assimilation, chlorophyll estimation, free proline content, K^+ and Na^+ content, as well as the activities of some enzymes including TPS, superoxide dismutase (SOD), betaine aldehyde dehydrogease (BADH), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) (Jia *et al.,* 2011). Each of these parameters exhibits special mechanisms. For the different plants in various environmental conditions, the salt-resistance of plant may be mediated by different parameters or a coordination of different parameters. Therefore, the practical salt-resistance of plant is really a complicated event, and the successfully transformation and preliminary expression of the *PyTPS* gene in rice is only the start for understanding the complex mechanism and for practical breeding of salt-tolerance in plant. In addition, the 3 obtained *TPS* transgenic rice lines showed resistance to 8‰ NaCl solution, which is still far lower to the ordinary sea water salinity (30-32‰), there are a lot of work to be done before the transgenic lines can be used in practical production.

Trehalose accumulation and salt-stress tolerance of the *TPS* transgenic plants: The increase of trehalose accumulation in *TPS* transgenic plants could explain the enhanced stress capacity was a result of the expression of the exotic transformed *TPS* gene. Increased trehalose accumulation has been reported in *TPS* gene transgenic rice (Garg *et al.,* 2002; Jang, 2003), tobacco (Holmstrom *et al.,* 1996; Romero *et al.,* 1997; Pilon-Smits *et al.,* 1998; Karim *et al.,* 2007) and potato (Goddijn *et al.,* 1997). Similarly, we found increase in trehalose content in *PyTPS* gene transgenic rice lines. This result strongly supports the fact that the *PyTPS* gene has been successfully transformed and expressed in the transgenic rice lines. Comparing with the normal inhabitant salinity (30-32‰) of *P. yezoensis,* the transgenic rice lines merely resistant to 8‰ NaCl solution, therefore it is considered that some other factors may be related to the mechanism of salt-resistance in rice.

Detection of the transgene in the offspring of transgenic lines: Segregation and silencing of the transgene often occur in transgenic plants. Here, in our study, much attention was paid to the identification of transgenic plants and the selection of stable transgenic plants. PCR identification was performed in every generation with the primer-pair P1/P2, and with all of 3 primer-pairs in the key generation T_1. The obtained 3 high salt-tolerance T_2 transgenic lines were further identified and selected by PCR, Southern hybridization, northern hybridization and RT-PCR analyses. It is showed that the *PyTPS* gene had integrated into the genome DNA of the transgenic rice plants and expressed indeed. In genetic transmission tests, the transformed *PyTPS* gene was absent in a few individuals in the T_0 and T_1 generations and only absent in one of the 10 assayed individuals in the T_2 generation, but it was detected in all of the assayed transgenic rice plants from the T_3 to the T_6 generations. This indicated that the transgene *PyTPS* can be transmitted from parent to offspring in transgenic rice plants, and the transgenic lines are closing to stable and homozygous after the T_3 generation.

REC of the salt-tolerant transgenic rice lines: REC is an important function indicator of cellular function. In high salt conditions, changes in plant cellular permeability will lead to increased REC (Sukumaran and Weiser, 1972; Liu *et al.,* 2009). Here in our study, REC determinations indicated that both transgenic lines and the non-transformed control line exhibited increased REC level after treatment with salt solutions, and that the higher the salt concentration, the higher the REC level obtained (Fig. 6). When treated with the same concentration of salt solution, the increased REC level of transgenic line was much less than that of the non-transformed controls. The transgenic lines suffered much less damage than the non-transformed controls under salt stress conditions; this suggests that the salt-tolerance was enhanced.

Yield evaluation: Considering the facts that the transgenic lines have not got release permission, and the fact that in practical rice production in saline-alkali soil, the planted rice is not salt stressed for its whole life history like seaweed, usually the planted rice plants will be irrigated with fresh-water and rain falls for numbers of times during growing season. We designed the pot-planting combined with fresh-water and salty-water alternative irrigation method to evaluate the yield preliminarily. The obtained data at least give us a comparison between the transgenic plants and the non-transformed control plants, which will help us to make decision in selection.

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