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Research Article

Physiological and Molecular Features of *Puccinellia tenuiflora* Tolerating Salt and Alkaline-Salt Stress

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Abstract

Saline-alkali soil seriously threatens agriculture productivity; therefore, understanding the mechanism of plant tolerance to alkaline-salt stress has become a major challenge. Halophytic *Puccinellia tenuiflora* can tolerate salt and alkaline-salt stress, and is thus an ideal plant for studying this tolerance mechanism. In this study, we examined the salt and alkaline-salt stress tolerance of *P. tenuiflora*, and analyzed gene expression profiles under these stresses. Physiological experiments revealed that *P. tenuiflora* can grow normally with maximum stress under 600 mmol/L NaCl and 150 mmol/L Na_2CO_3 (pH 11.0) for 6 d. We identified 4,982 unigenes closely homologous to rice and barley. Furthermore, 1,105 genes showed differentially expressed profiles under salt and alkaline-salt treatments. Differentially expressed genes were overrepresented in functions of photosynthesis, oxidation reduction, signal transduction, and transcription regulation. Almost all genes downregulated under salt and alkaline-salt stress were related to cell structure, photosynthesis, and protein synthesis. Comparing with salt stress, alkaline-salt stress triggered more differentially expressed genes and significantly upregulated genes related to H^+ transport and citric acid synthesis. These data indicate common and diverse features of salt and alkaline-salt stress tolerance, and give novel insights into the molecular and physiological mechanisms of plant salt and alkaline-salt tolerance.

Keywords: *Puccinellia tenuiflora*; halophyte; salt and alkaline-salt tolerance; microarray assay; gene expression profiles.

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Introduction

Salt stress and alkaline-salt stress are important abiotic stress factors that greatly affect growth and development of plants and severely threaten agricultural productivity (Greenway and Munns 1980; Campbell and Nishio 2000; Degenhardt et al. 2000; Zhu 2002; Bhatnagar-Mathur et al. 2008). The United Nations Educational, Scientific and Cultural Organization and the Food and Agriculture Organization of the United Nations statistics show that the saline-alkali soil area in the world has reached 950 million ha, and saline-alkali erosion will lead to increased loss of arable land in the next few years, with up to 50% loss by 2050 (Wang et al. 2003). Both salt and alkali pH stress in alkaline-salt soil retard the growth of plants or kill plants. Therefore, understanding the mechanism of plant tolerance to alkaline-salt stress and establishing molecular biotechniques to engineer stress-tolerant plants have been focuses in the plant and agriculture sciences.

Numerous studies have revealed several important molecules and signaling pathways involved in saline tolerance of plants, such as the Salt Overly Sensitive (SOS) pathway and components of the pathway in *Arabidopsis thaliana*, which have

the potential for use to improve the productivity of plants (Zhu 2001b; Chinnusamy et al. 2006; Zhang et al. 2012). Relative to single salt stress, alkaline-salt has a combined harm of salt and alkaline pH. The molecular features and mechanisms of plants resistant to alkaline-salt stress are largely unknown. The present studies have mainly focused on model plants such as *A. thaliana* and *Oryza sativa* (Zhu 2000; Kawasaki et al. 2001; Zhou and Wang 2002; Sahi et al. 2006), which are glycophytes and do not have a strong phenotype resistant to salt stress. Therefore, halophytes, such as *Thellungiella halophila*, which is closely related to *A. thaliana*, have been proposed as model plants for studying the molecular mechanisms of salinity tolerance (Gong et al. 2005; Ghars et al. 2008; Pang et al. 2010).

Puccinellia tenuiflora is a monocotyledonous halophyte distributed in the northeast of China. It belongs to the same genus as rice, Gramineae. Compared with other halophytes, *P. tenuiflora* can grow normally in saline soil and under pH 9 (Wan and Zou 1990). Thus, *P. tenuiflora* may be an ideal material for studying the mechanisms of salt and alkali tolerance of plants. Several studies have revealed the salt-tolerant phenotype of *P. tenuiflora* associated with the ability of the plant to selectively uptake K^+ (Peng et al. 2004; Wang et al. 2009) and exude salt through the stomata of leaves (Sun et al. 2005). The genes functionally involved in salt tolerance were found to be *PutHKT2;1* (Ardie et al. 2009), *PutCAX1* (Liu et al. 2009), and *PutPMP3* (Zhang et al. 2008). Some studies, many of them Chinese, have reported the physiological features and gene expression files of *P. tenuiflora*. Yu et al. (2011) investigated the changes in biomass, inorganic ion content, osmolytes, photosynthesis, defense-related enzyme activities, and metabolites under 50 and 150 mmol/L NaCl treatments, respectively, and identified differentially expressed proteins responding to salt stress by a proteomic strategy. Another study (Kobayashi et al. 2012) compared the effect of salt (100, 300, and 1,000 mmol/L) and $NaHCO_3$ (100, 300, and 1,000 mmol/L) treatment on the growth of *P. tenuiflora* and rice by showing pictures of the treated plants, and analyzed the expression of two candidate genes *NHA* and *NHX* in *P. tenuiflora* and in rice. Many Chinese studies mainly focused on the physiological response for the tolerance of *P. tenuiflora* to salt and alkaline-salt. So far, there are only two studies (Wang et al. 2007b, c) which have reported gene expression of *P. tenuiflora* under stress. One generated a cDNA library from leaves treated with 450 mmol/L $NaHCO_3$, and sequenced a part of expressed sequence tags (ESTs) (Wang et al. 2007b), and the other went further to compare the 1,067 unigenes' expression of *P. tenuiflora* under 400 mmol/L $NaHCO_3$ for 6, 12, 24, and 48 h, and obtained 95 differentially expressed genes (Wang et al. 2007c). In addition, some data showed that the accumulation of citric acid was specific to alkali stress (Shi et al. 2002). However, all these studies mentioned above did not define several basic features related to salt and alkali tolerance of the plant, such as how much salt and alkali-salt the plant can tolerate, and whether response to salt and alkali stresses produces any differences in gene expression profiles. These issues are essential to further understand the mechanisms of tolerance to salt and alkaline-salt stress, and to identify more genes to engineer plants resistant to salt and alkaline-salt stress.

In our study, we investigated the ability of *P. tenuiflora* to tolerate 600 and 900 mmol/L NaCl and 150 and 200 mmol/L Na_2CO_3 (pH 11.0), which represent non-lethal and lethal levels of salt and alkali-salt stress, respectively. We sequenced ESTs of *P. tenuiflora* under stress, and searched for homologs. Finally, we analyzed the differential expression of genes under salt and alkaline-salt stress to reveal molecular features of the plant response to salt and alkaline-salt stress.

Results

Salt and alkaline-salt tolerance of *P. tenuiflora*

To identify the ability of *P. tenuiflora* to tolerate salt and alkaline-salt stress, we treated 45-d-old seedlings with concentrations of NaCl and Na_2CO_3 (pH 11.0) for 1–6 d, and then analyzed growth under stress and non-stress conditions (**Figure 1**). NaCl treatment for 6 d caused dehydration and wilting of treated seedlings with 300, 600, and 900 mmol/L NaCl, with the most severe phenotype occurring with the 900 mmol/L NaCl treatment (**Figure 1A**). However, after transfer to normal conditions for 10 d, the growth of seedlings treated with 300 and 600 mmol/L NaCl could be recovered, but seedlings with 900 mmol/L NaCl were nearly dead (**Figure 1A**). Similarly, treatment with Na_2CO_3 (pH 11.0) led to dehydration and wilting. With growth recovery, seedlings could tolerate up to 150 mmol/L Na_2CO_3 (pH 11.0) but not 200 mmol/L or more Na_2CO_3 (pH 11.0) (**Figure 1B**). In addition, under 50 mmol/L Na_2CO_3 (pH 11.0), *P. tenuiflora* exhibited growth even better than under normal conditions (**Figure 1B**), and after 6 d of such treatment, plants were shown to have no water loss, implying that a suitable concentration of alkaline could promote the growth of *P. tenuiflora*.

We further examined changes in the water content of seedlings treated with concentrations of NaCl or Na_2CO_3 (pH 11.0) (**Figure 1C, D**). The water content decreased quickly with increasing concentrations of NaCl and Na_2CO_3 (pH 11.0) (**Figure 1C, D**, Table S1). With growth recovery, the water content of seedlings treated with 600 mmol/L NaCl or 150 mmol/L Na_2CO_3 (pH 11.0) was 80% or more of the normal level after a 10 d recovery period, with no water content recovery in seedlings with 900 mmol/L NaCl and 200 mmol/L Na_2CO_3 (pH 11.0). This finding was consistent with the seedlings' ability to survive at 600 mmol/L NaCl or 150 mmol/L Na_2CO_3 (pH 11.0). Therefore, *P. tenuiflora* can tolerate high levels of

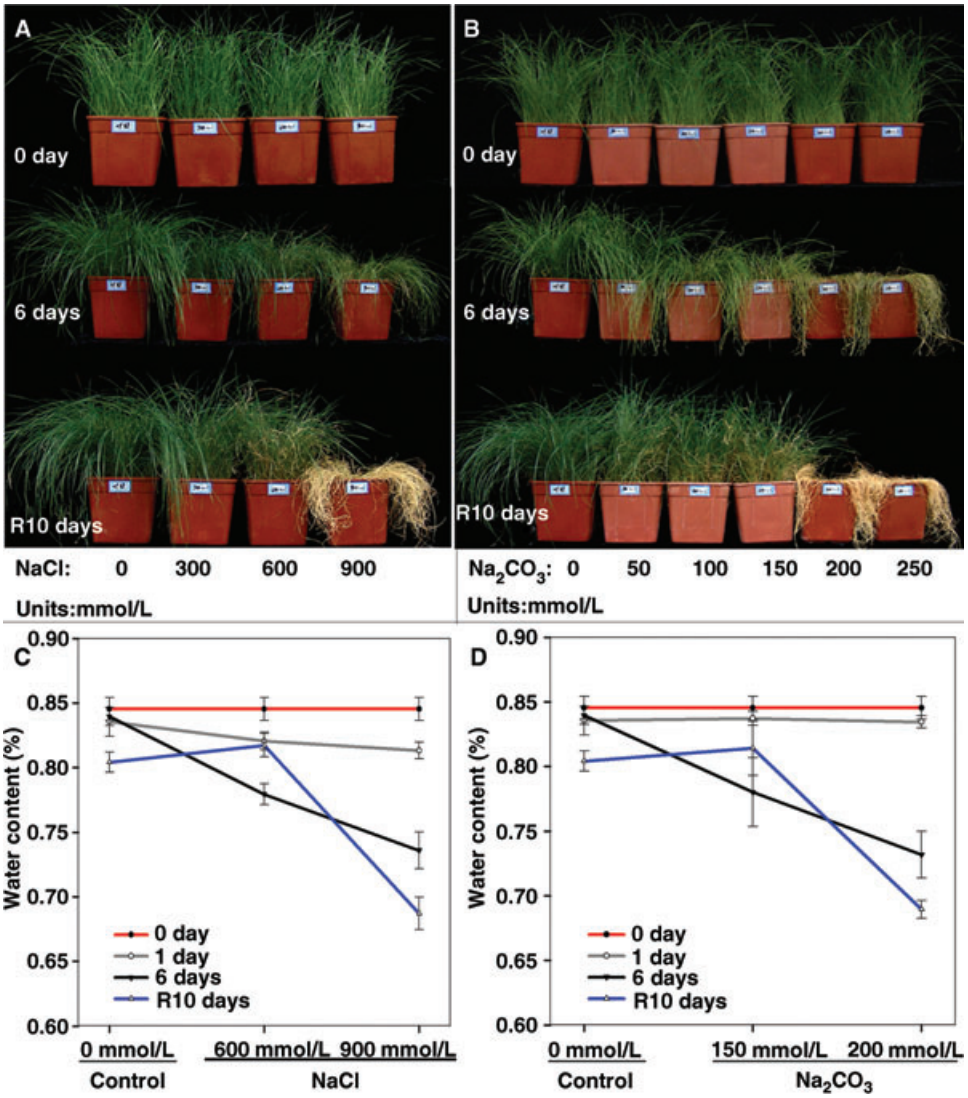

Figure 1. Physiological characteristics of *Puccinellia tenuiflora* under salt and alkaline-salt treatment.

Forty-five-d-old seedlings were treated with different concentrations of NaCl **(A)** and Na_2CO_3 (pH 11.0) **(B)** for 0 d and 6 d, then underwent growth recovery for 10 d. The corresponding water content is shown in **(C)** under NaCl and in **(D)** under Na_2CO_3 (pH 11.0). R 10 days, recovery for 10 d.

salt and alkaline-salt. The conditions of 600 mmol/L NaCl and 150 mmol/L Na_2CO_3 (pH 11.0) were considered to be the maximal levels of salt and alkaline-salt, respectively, that the plant could tolerate in experimental conditions.

Characterization of ESTs

To generate a set of mRNAs as complete as possible, we constructed a cDNA library of *P. tenuiflora* seedlings treated with moderate strength 100 mmol/L Na_2CO_3 (pH 11.0) stress. The cDNA library had a titer of 1.5×10^5 plaque-forming units, the recombinant percentage was close to 100%, and the insert cDNA fragment of 1–3 kb agreed with the average insert size of more than 1 kb.

By sequencing cDNA fragments of the library, we obtained 11,039 ESTs, with 8,361 being of high quality. After assembly and alignment, the dataset generated 4,982 unigenes (Table S2) with an average length of 483.94 bp. According to the Clusters of Orthologous Groups of proteins (COGs) (Tatusov et al. 2000; Tatusov et al. 2001) database, 1,249 of the 4,982 unigenes had annotated function information and were categorized into 24 functional groups, with preferred distribution in six major groups that contained approximately two-thirds of all COG annotated

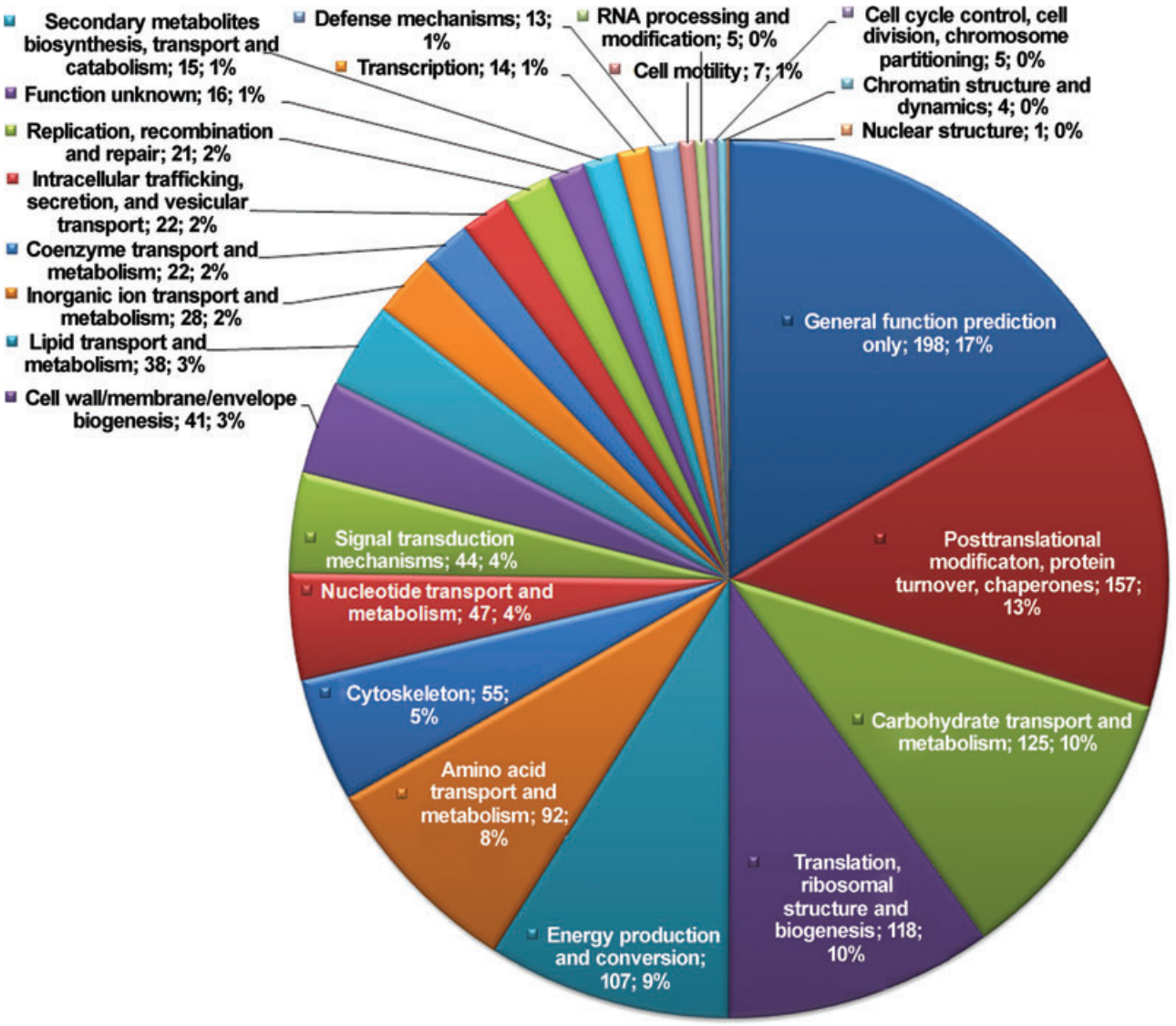

Figure 2. Functional categories of unigenes by Clusters of Orthologous Groups (COGs) annotation.

unigenes (Figure 2). The six groups – (i) general function prediction only; (ii) post-translational modification, protein turnover, and chaperones; (iii) translation, ribosomal structure, and biogenesis; (iv) carbohydrate transport and metabolism; (v) energy production and conversion; and (vi) amino acid transport and metabolism – accounted for 18.49%, 13.29%, 10.65%, 9.05%, 8.49%, and 7.37% of all COG annotated unigenes, respectively.

Using a BlastNt homology search against the EST databases for other green plants, with a cut-off of 1e5, we identified the homologs of the 4,982 unigenes. Thirty-three percent (1,643) of these unigenes had homologs in *O. sativa*, and 28.6% (1,424) in *Hordeum vulgare* (Figure 3), which implies a close genetic relationship between *P. tenuiflora*, *O. sativa*, and *H. vulgare*.

Identification and characterization of differentially expressed genes responsive to salt and alkaline-salt stress

To understand the molecular mechanism of *P. tenuiflora* tolerating salt and alkaline-salt stress, we identified genes responsive to these stresses through a microarray assay with the 4,982 unigenes described above (Tables S3, S4). Hybridization with labeled RNA samples from seedlings treated with salt and alkaline-salt solutions revealed 1,105 genes with differential expression of more than twofold under salt and alkaline-salt stress (Figure 4A, Table S5). Among these genes, 383, 331, 374, and 799 showed changed expression in response to 600 and 900 mmol/L NaCl and 150 and 200 mmol/L

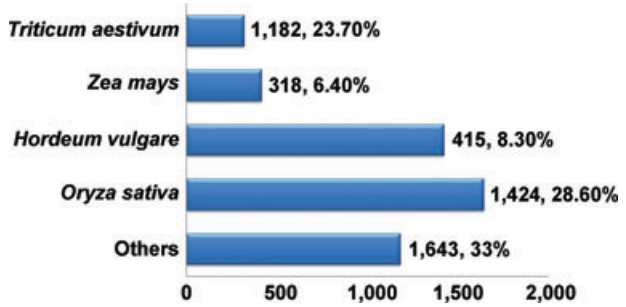

Figure 3. Homologs of *Puccinellia tenuiflora* unigenes in other plants.

The percentage in the histogram represents the number of genes in each species with homology to *P. tenuiflora* unigenes. Number of unigenes is on the x-axis.

Na_2CO_3 treatments, respectively (Figure 4B). The number of upregulated genes was increased with an increasing concentration of both salt and alkaline-salt (Table 1). However, the number of downregulated genes decreased from 262 to 142 with an increasing NaCl concentration, and increased from 260 to 510 with an increasing Na_2CO_3 concentration (Table 1). Furthermore, among these genes, 76 and 19 appeared to specifically respond to 600 mmol/L NaCl and 150 mmol/L Na_2CO_3, respectively, and 183 and 368 specifically responded to 900 mmol/L NaCl and 200 mmol/L Na_2CO_3, the lethal doses of salt and alkaline-salt stress, respectively (Figure 4).

Further functional annotation by a BlastX search in nr databases (Altschul et al. 1990) with a cut-off of 1e5 revealed that the differentially expressed genes were organized into 13 functional categories (Table 1, Table S6) (Bohnert et al. 2001). The groups metabolism, signal transduction, transcription, and cell rescue and defense were overrepresented. Almost all genes involved in cell structure, photosynthesis and protein synthesis were downregulated with salt and alkaline-salt stress (Table 1). Of particular note, we did not detect cell structure-related genes with a changed expression under 600 mmol/L NaCl and 150 mmol/L Na_2CO_3 treatments, but these genes were downregulated under lethal doses (Figure 5A). In addition, genes involved in citric acid synthesis and H^+-ATPase transport showed a Na_2CO_3 dose-dependent increase, which was more upregulated than with NaCl stress (Figure 5B, C).

We annotated 598 of the 1,105 genes with differential expression by gene ontology (GO) (Table S7), and found that the GO terms of photosynthesis, oxidation reduction, oxidoreductase activity, microtubule and translation were overrepresented (Figure 6).

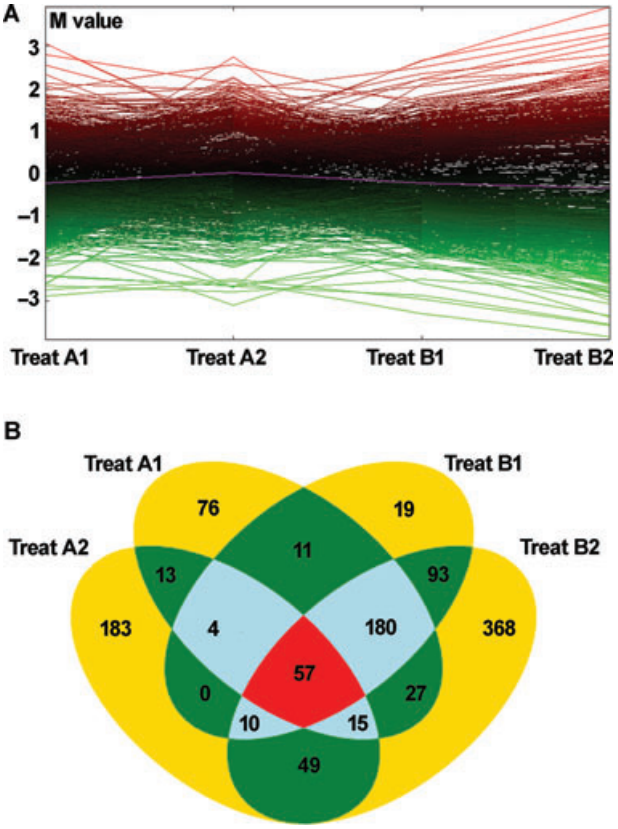

Figure 4. Expression patterns of differentially expressed genes and their distribution with the salt and alkali-salt stress.

(A) Expression patterns.
(B) Distribution of genes with the four treatments. Treat A1, 600 mmol/L NaCl; Treat A2, 900 mmol/L NaCl; Treat B1, 150 mmol/L Na_2CO_3 (pH 11.0); Treat B2, 200 mmol/L Na_2CO_3 (pH 11.0).

Reliability analysis of cDNA microarray data

We hybridized 16 microarrays in this study and found a high correlation ($r \geq 0.88$) for fluorescence intensity with each pair hybridized under a given stress (Table 2). In addition, the technical correlation for the four different treatments was high ($R^2 \geq 0.88$) (Figure 7A). This indicated the reliability of the microarray hybridization data. Furthermore, we used quantitative reverse transcription polymerase chain reaction (RT-PCR) with randomly selected, differentially expressed genes, and found that the expression profiles detected by microarrays were in good agreement with the RT-PCR results (Figure 7B, Table S8).

Discussion

Using a physiological approach, a cDNA library analysis, and EST sequencing, we have revealed the physiological features

Table 1. Distribution of 1,105 differential expression genes with salt and alkali-salt treatment in 13 functional groups

		NaCl				Na_2CO_3			
		600 mmol/L		900 mmol/L		150 mmol/L		200 mmol/L	
Classification	No. of unigenes	Up	Down	Up	Down	Up	Down	Up	Down
Cell growth, division	27	1	5	3	3	2	7	8	15
Cell rescue, defense	91	17	17	22	19	15	17	27	27
Cell structure	15	0	0	1	7	0	0	1	12
Metabolism	169	19	39	35	15	22	27	61	65
Energy	47	9	9	7	4	8	5	22	15
Photosynthesis	37	1	23	0	11	1	23	1	30
Protein destination	84	6	23	14	3	10	24	30	34
Protein synthesis	62	2	18	0	0	2	15	4	52
Signal transduction	110	10	20	35	21	11	23	31	36
Transcription	93	5	22	13	9	8	22	19	52
Transport facilitation	57	7	9	14	2	8	10	23	22
Unknown	153	16	36	21	19	15	48	37	79
No hit	160	28	41	24	29	12	39	25	71
Total	1,105	121	262	189	142	114	260	289	510
		383		331		374		799	

Corresponding information can be found in Supporting Information in Table S5. "Up" means upregulated genes, "Down" means downregulated genes.

of *P. tenuiflora* in response to saline and alkaline-salt stress. We identified 4,982 unigenes from the plant associated with alkali-salt stress. A further microarray assay identified 1,105 genes with differential expression in response to salt and alkaline-salt stress, and we revealed the functional characteristics of these genes. Our results provide novel physiological and molecular information related to the salt and alkaline-salt tolerance of *P. tenuiflora*, which are important in further understanding how plants tolerate salt and alkaline-salt stress.

Physiological and molecular features of *P. tenuiflora* in tolerating salt and alkaline-salt stress

As a halophytic plant, *P. tenuiflora* has a strong ability to tolerate salt and alkaline-salt stress (Yan et al. 1999); however, how much salt or salt in an alkali environment this plant can tolerate was previously unknown. We found that the plant can tolerate stress up to 600 mmol/L NaCl or 150 mmol/L Na_2CO_3 (pH 11.0), and this strong tolerance was associated with the physiological recovery of water content during recovery growth from the stress condition to the normal condition (**Figure 1**). Furthermore, an EST homology search revealed that *P. tenuiflora* is close to rice and barley (**Figure 3**), even though these species exhibit less salt and alkaline-salt tolerance. Studies of cereals have revealed that barley is more tolerant than rice, but still shows cessation of growth or development with 300 mmol/L or more NaCl (Munns and Tester 2008). *P. tenuiflora*, as a halophytic relative of rice and barley, is one of the most tolerant species to salt and alkaline-salt stress; therefore, especially among monocotyledon plants, it is an ideal model for understanding the mechanisms of salt and alkaline-salt tolerance.

In general, the stress tolerance phenotype of plants involves an integrated input of changed metabolism and cellular processes adapting to this stress, which requires the coordinated expression of genes involved in these processes and the regulation of gene expression and signaling (Yamaguchi-Shinozaki and Shinozaki 2006). Among our 1,105 genes with differential expression, those (except for the unknown and no hit genes) involved in metabolism, cell rescue and defense, photosynthesis, signal transduction and transcription were overrepresented (**Table 1**). Consistent with the observation that the greatest response to salinity is decreased photosynthesis (Munns and Tester 2008), almost all of the genes implicated in photosynthesis were downregulated with all four treatments, as exemplified by genes encoding chlorophyll a/b binding protein, light-harvesting complex IIa protein, photosystem II oxygen-evolving complex protein PsbP family protein, ribulose-1,5-bisphosphate carboxylase/oxygenase, and ferredoxin-NADP(H) oxidoreductase (**Table 1** and Table S6). In general, the reduced rate of photosynthesis is followed by the increased generation of reactive oxygen species and the increased activity of detoxifying enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, and diverse peroxidases (Apel and Hirt 2004; Logan et al. 2006). Our data showed that genes with oxidation reduction and oxidoreductase

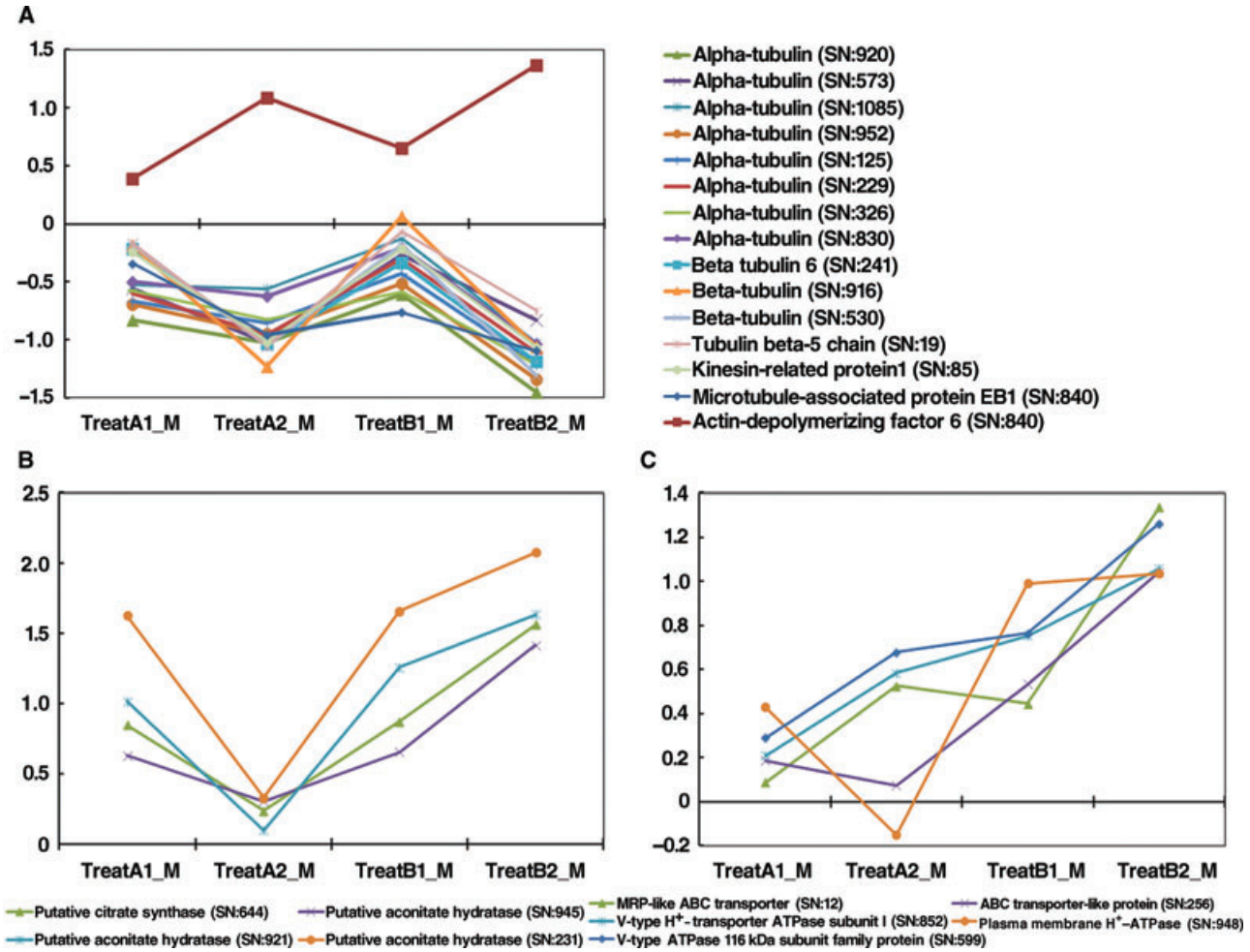

Figure 5. Patterns of gene expression under salt stress and alkaline-salt stress.

(A) Cell structure-related genes.

(B) Genes related to citric acid synthesis.

(C) H^+-ATPase transporter-related genes.

SN, series number; treat A1, 600 mmol/L NaCl; treat A2, 900 mmol/L NaCl; treat B1, 150 mmol/L Na_2CO_3 (pH 11.0); treat B2, 200 mmol/L Na_2CO_3 (pH 11.0).

activity functions were major differentially expressed genes, and most of these genes were upregulated in treated conditions (**Figure 6**, Table S7). These results suggest that photosynthesis and redox homeostasis are both important for plants to handle salt and alkaline-salt stress.

Recently, microfilament (MF) and microtubulin (MT) were confirmed to be closely related to the salt stress response (Wang et al. 2011). In *Arabidopsis*, the salt-tolerant phenotype depends on MT reorganization (Wang et al. 2007a) and requires MF polymerization, whereas lethally high salt stress causes MF disassembly (Wang et al. 2010a). Consistently, we also found that a lethal dose of NaCl and Na_2CO_3 (pH 11.0) upregulated genes encoding actin-depolymerizing factor 6, and downregulated all MT-related genes (**Figure 5A**, Table S6). In addition to the cytoskeleton-related genes, osmotic homeostasis is also essential for normal cell functions. Our study revealed that salt and alkaline-salt stress mediate the expression change of genes involved in osmolyte biosynthesis and transportation, including proline synthesis, sugar and proline transporters, water channel proteins, ion channels, and transport ATPases (Table S6), which are known to function in stress tolerance (Yamaguchi-Shinozaki and Shinozaki 2006; Munns and Tester 2008). Thus, the dynamic regulation of the genes involved in the cytoskeleton and osmotic homeostasis.

Signal transduction pathways and transcription factors function in regulating stress-responsive gene expression for plant

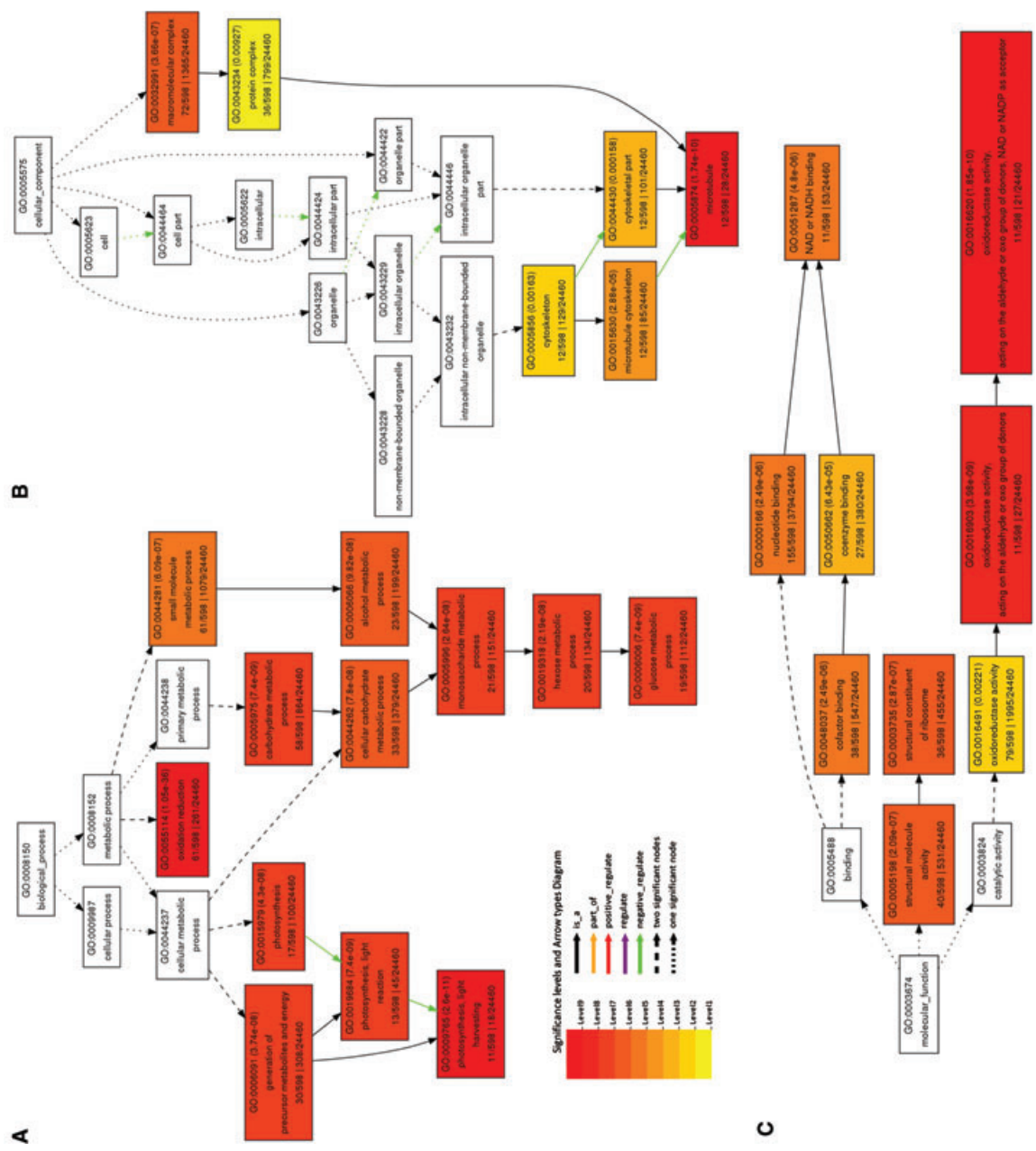

Figure 6. Gene ontology (GO) terms for 1,105 genes with differential expression under salt stress and alkaline-salt stress.

Genes with GO terms for **(A)** biological processes, **(B)** cellular component, and **(C)** molecular function branches. The classical terms and their serial numbers are represented as rectangles. For significant terms, the rectangle includes the GO terms, adjusted *P*-value (in parentheses), item number mapping the GO term in query list and background, and the total number of items in the query list and background. The color scale shows the *P*-value cut-off levels for each biological process; the greater the statistical significance, the darker and redder the color.

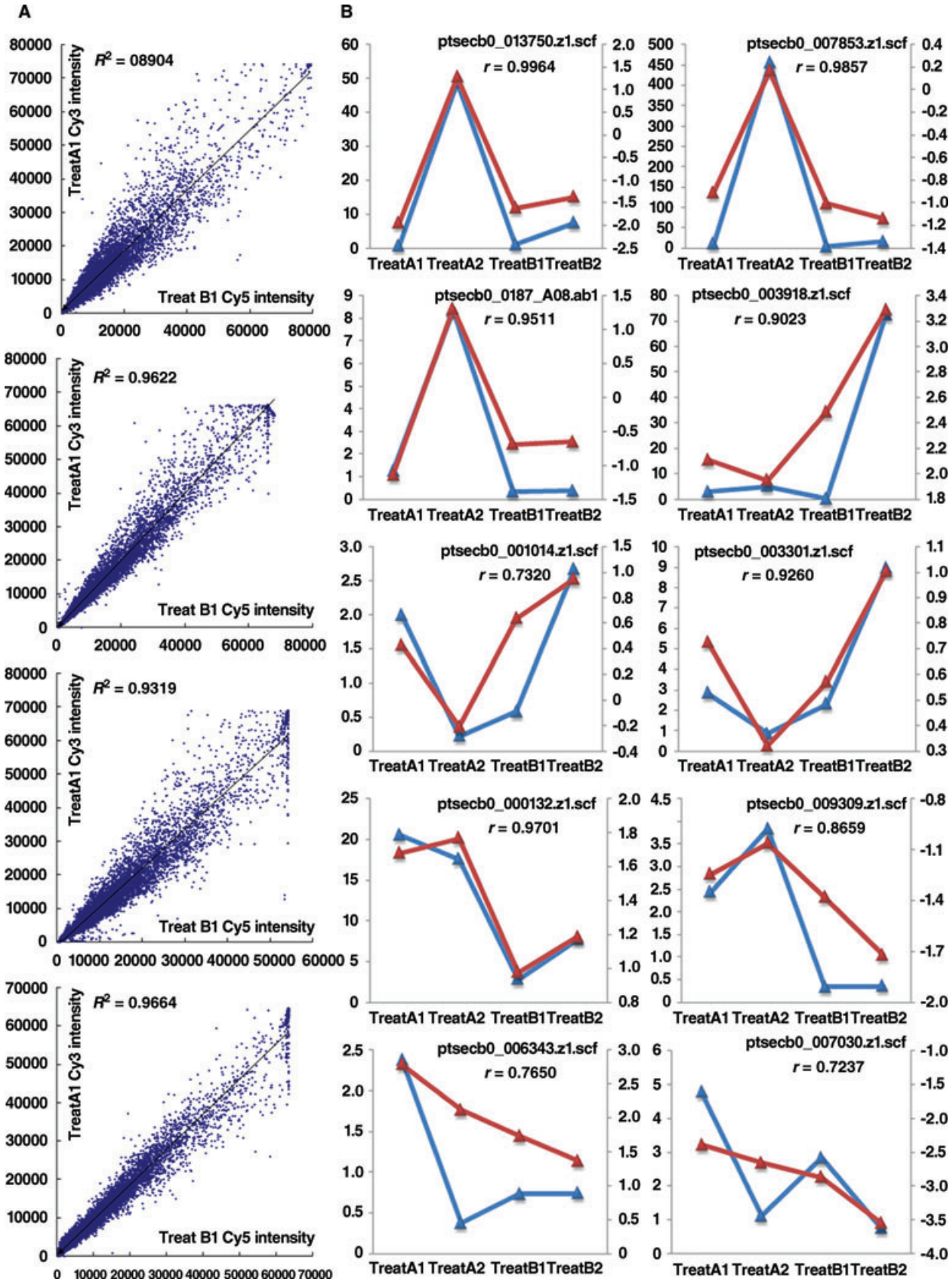
A
B
R^2 = 08904
R^2 = 0.9622
R^2 = 0.9319
R^2 = 0.9664
TreatA1 Cy3 intensity
Treat B1 Cy5 intensity
ptsecb0_013750.z1.scf
r = 0.9964
ptsecb0_007853.z1.scf
r = 0.9857
ptsecb0_0187_A08.ab1
r = 0.9511
ptsecb0_003918.z1.scf
r = 0.9023
ptsecb0_001014.z1.scf
r = 0.7320
ptsecb0_003301.z1.scf
r = 0.9260
ptsecb0_000132.z1.scf
r = 0.9701
ptsecb0_009309.z1.scf
r = 0.8659
ptsecb0_006343.z1.scf
r = 0.7650
ptsecb0_007030.z1.scf
r = 0.7237
TreatA1 TreatA2 TreatB1TreatB2

Table 2. Correlation coefficients for fluorescence intensity between triplicate microarray hybridization experiments under each stress

Treatments		Correlation coefficient (*r*)		
		1–2	1–3	2–3
A1	NaCl, 600 mmol/L	0.8871	0.9248	0.9501
A2	NaCl, 900 mmol/L	0.9600	0.9681	0.9712
B1	Na_2CO_3, 150 mmol/L	0.9362	0.9256	0.9291
B2	Na_2CO_3, 200 mmol/L	0.9492	0.9519	0.9574

adaptation to environmental stresses (Yamaguchi-Shinozaki and Shinozaki 2006). Ca^{2+}, a second messenger, modulates intracellular Na^+ homeostasis and salt tolerance (Zhu 2002; Lin et al. 2009), and is activated by an SOS signaling pathway, a known signaling system in the salt stress response (Zhu et al. 1998; Zhu 2001a). Genes of most SOS system members, such as calcineurin B-like protein (CBL/SOS3), CBL-interacting protein kinase (CIPK/SOS2), and Na^+/H^+ antiporter (SOS1), showed changed expression with salt and alkaline-salt stress (Table S6) (Zhong et al. 2012). Several studies have demonstrated that salt stress can activate the cell signaling pathway via protein phosphorylation of some protein kinases which are central players (Boudsocq and Lauriere 2005; Kodama et al. 2009). We found salt- and alkaline-salt-responsive kinase genes overrepresented among signal-transduction genes (Table S6). For example, those encoding receptor-like protein kinase, serine/threonine protein kinase, phosphatidylinositol 3,5-kinase, mitogen-activated protein kinase, and protein phosphatase 2C, were all shown to be involved in salt tolerance (Jonak et al. 1996; Kiegerl et al. 2000; Meskiene et al. 2003; Shiu and Bleecker 2003). In addition, transcription regulation is an important mechanism for plants to adapt to abiotic stresses (Century et al. 2008; Santos et al. 2011), and studies have shown that the zinc finger family proteins, MYB, MYC, and hormone-responsive transcription factors including abscisic acid-responsive proteins and auxin response factors, are all important factors in regulating plant stress tolerance (Yamaguchi-Shinozaki and Shinozaki 1994; Mukhopadhyay et al. 2004; Wang et al. 2008; Wang et al. 2010b). We found hormone-responsive regulators upregulated with salt and alkaline-salt stress, but most of the other transcription factors were downregulated (Table S6), which implies the importance of kinase-mediated signaling and transcription regulation in salt and alkaline-salt tolerance of plants.

Differences in response to salt and alkali-salt stresses

The ability of *P. tenuiflora* to tolerate salt and alkaline-salt allows us to dissect differences in plants in response to salt and alkaline-salt stress. A reciprocal enhancement of salt and alkali stress is characteristic of alkaline-salt mixed stress (ElSamad and Shaddad 1996); however, a comparison of gene expression patterns responsive to salt and alkaline-salt stress is needed to identify genes mainly responsive to each of the two types of stresses, in order to understand the molecular mechanisms by which plants handle salt and alkaline-salt stress individually. Physiological experiments have revealed that *P. tenuiflora* can tolerate up to 600 mmol/L NaCl but only 150 mmol/L Na_2CO_3. In other words, the tolerance concentration of Na^+ was decreased by half in a high-pH environment. We found a similar number of genes (~380) with a changed expression under 600 mmol/L NaCl and 150 mmol/L Na_2CO_3 (**Table 1**). With the concentration of salt and alkaline-salt increased to a lethal dose, the number of genes with differential expression under alkaline-salt stress was more than twice that of those under salt stress (**Table 1**). Furthermore, compared with survival of salt and alkaline-salt stress, the lethal Na_2CO_3 stress significantly increased the number of genes with upregulated and downregulated expression, whereas lethal salt stress led to an increased number of upregulated genes but a greatly decreased number of downregulated genes. Impressively, our study revealed genes specifically responding to salt or alkaline-salt (**Table 1**). The stress-specific genes were only a small portion of those genes with differential expression under a tolerant level of salt and alkaline-salt, but significantly increased as levels of salt and alkaline-salt content up to the lethal dose. The number of stress-specific genes with the lethal dose of alkaline-salt (368 genes) were twice that of those with a

Figure 7. Assessment of the microarray data quality.

(A) Scatter plot of dye-swap experiment; R^2, correlation coefficient between Cy3 and Cy5 fluorescent intensity under each stress treatment. **(B)** Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmation of expression profiles for genes with differential expression. The x-axis shows the four different treatments, and the y-axis shows the expression level from microarray data (left) and quantitative (q)RT-PCR (right). The blue line represents expression profiles by qRT-PCR, and the red line represents the expression profiles by microarray assay. *r*, correlation coefficients for gene expression results between quantitative RT-PCR and microarray analysis during a certain stress treatment. Treat A1, 600 mmol/L NaCl; Treat A2, 900 mmol/L NaCl; Treat B1, 150 mmol/L Na_2CO_3 (pH 11.0); Treat B2, 200 mmol/L Na_2CO_3 (pH 11.0).

lethal dose of salt (183 genes) (**Figure 4B**). Thus, alkaline-salt stress may have a greater effect on gene expression than salt stress, which is consistent with previous reports that a high-pH environment has a great influence on plant growth and development (Arnon et al. 1942; Tang et al. 1993; Peng et al. 2008).

Alkaline-salt stress has a complex effect on plant growth and development via osmotic stress and high pH stress (Yang et al. 2009). The alkali pH stress affects plant growth and development mainly by modifying the intracellular pH environment (Lager et al. 2010). Several studies have demonstrated H^+ transporter-related genes, such as plasma membrane H^+-ATPase and V-type H^+-transporting ATPase, to be involved in salt tolerance and to be positively regulated by salt stress (Caracuel et al. 2003; Shen et al. 2011). Our data showed that when compared with salt stress, alkaline-salt stress differentially regulated more H^+ transporter-related genes, with higher levels of expression for upregulated genes (**Figure 5C**). Thus, transporters may be important in regulating intracellular pH homeostasis under alkali stress. Furthermore, genes related to citric acid synthesis were differentially regulated in response to salt and alkaline-salt stress. These genes were upregulated with the increasing strength of alkaline-salt stress, but were downregulated under high salt content (**Figure 5B**). The accumulation of citric acid is a specific physiological response to alkaline stress in *P. tenuiflora*, and accumulated citric acid plays an important role in the maintenance of pH balance under stress (Shi et al. 2002). Therefore, the increased ability to regulate intracellular pH homeostasis and synthesize citric acid may be an important feature for these plants to cope with alkali-salt stress.

Materials and Methods

Plant materials and physiological treatments

Seeds of *Puccinellia tenuiflora* (Griseb.) Scribn. et Merr. were transplanted in pots containing vermiculite irrigated with Hoagland solution. Seedlings were grown in a greenhouse under 70% relative humidity, and under 12:12 h light:dark conditions at 28 °C/25 °C. Forty-five-d-old seedlings were treated with Hoagland solution containing 300, 600, or 900 mmol/L NaCl, or 50, 100, 150, 200, and 250 mmol/L Na_2CO_3 (pH 11.0). After 0, 1, and 6 d, a set of approximately 10 young seedlings was harvested randomly in each pot to measure fresh weight (FW) and dry weight (DW). The remained seedlings were grown in Hoagland solution for a recovery growth period of 10 d, and were then randomly selected for FW and DW measurements. DW was determined after dehydration at 105 °C for 10 min and desiccation at 80 °C to a constant weight. The water content (WC) of seedlings was estimated as WC = (FW – DW) / DW. For each treatment, three biological replicates were performed.

For cDNA library construction, 45-d-old seedlings treated with 100 mmol/L Na_2CO_3 in Hoagland solution (pH 11.0) for 24 h were harvested and stored at –80 °C. To prepare target RNAs for microarray hybridization, 45-d-old seedlings treated for 12 h with Hoagland solution containing 600 or 900 mmol/L NaCl or 150 or 200 mmol/L Na_2CO_3 (pH 11.0) were harvested and stored at – 80 °C. Each treatment involved three biological replicates. All controls were grown in normal Hoagland solution under the same conditions.

RNA extraction

Total RNA from seedlings was isolated by the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA). The yield and quality of total RNA was determined by spectrophotometry (DU730; Beckman Coulter, Brea, CA, USA) and electrophoresis under an agarose EtBr gel. Poly (A) $^+$ RNA was isolated with the use of an Oligotex mRNA Kit (Qiagen, Hilden, Germany), and the quality of isolated mRNA was examined by spectrophotometry and electrophoresis.

cDNA library construction and EST sequencing

A cDNA library was constructed with use of the Creator SMART cDNA Construction Kit (Clontech, Mountain View, CA, USA). To obtain a high-quality cDNA library, double-strand cDNA was normalized with use of the Trimmer-Director kit (Evrogen, Moscow, Russia). cDNA fragments between 1 and 3 kb were collected with use of the QIAquick Gel Extraction Kit (Qiagen), ligated to the pDNR-LIB vector (Clontech), and then transformed into *Escherichia coli* DH10B by electroporation, and incubated on selective Luria–Bertani plates overnight at 37 °C. The quality of the cDNA library was assessed by titer, average insert size, and percentage recombinants.

Colonies containing cDNA fragments in the cDNA library were randomly selected, and insert fragments were sequenced with use of MegaBACE 1000 (Amersham Pharmacia, Piscataway, NJ, USA) to generate ESTs. The primer used for sequencing was the T7 forward primer (5′-TAATACGACTCA CTATAGGG-3′). Row EST sequence files were created with the use of Phred base-calling with Q20 criterion. After eliminating vector and polyA sequences, clean ESTs were obtained to assemble unigenes by the cap3 procedure. All the clean EST sequences have been deposited in the National Center for Biotechnology Information (NCBI) dbEST database (Library Access No. LIBEST_028028; EST sequence access No. JZ098916–JZ107276; www.ncbi.nlm.nih.gov/dbEST/index.html). Unigenes were annotated with a cut-off of 1e-5 by BLAST searches against the databases NCBI Nt (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PRO

GRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=&LINK_LOC=blasttab), NCBI Nr (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&BLAST_PROGRAMS=blastx&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=&LINK_LOC=blasttab), SWISSPROT (www.uniprot.org/), KEGG (www.genome.jp/kegg/), and COG (http://www.ncbi.nlm.nih.gov/COG/). The GO terms for unigenes were annotated through use of InterProScan ver. 4.5 (www.ebi.ac.uk/InterProScan/).

Microarray preparation, hybridization, and data analysis

cDNA fragments corresponding to all unigenes were spotted onto slides in adjacent duplicates by use of SpotArray 72 (Perkin Elmer, Cambridge, MA, USA). To control the quality of microarray preparation, we designed six internal standard genes, including three positive genes and three negative genes (Table S3). One positive and one negative gene were paired and distributed in a diagonal position on each small matrix. Each small matrix had three internal standard combinations. Before hybridization with target RNAs, control target RNA samples were labeled with the fluorochrome cyanine-3 (Cy3) and treated target RNAs were labeled with the fluorochrome cyanine-5 (Cy5). Dye swapping was performed between the control target RNAs and one of the triplicate-treated target RNAs. In total, 16 cDNA microarrays were hybridized with probes from four treatments, with each treatment including three biological replication samples and one dye-swap sample. The hybridization system was incubated at 42 °C for more than 16 h. Then, slides were washed two times in washing buffer I (2 × saline-sodium citrate (SSC) buffer + 0.1% sodium dodecylsulfate (SDS)) for 5 min each, two times in washing buffer II (0.2 × SSC + 0.1% SDS) for 5 min each, and two times in washing buffer III (0.2 × SSC + 0.1 mmol/L dithiothreitol) for 5 min each, then dried in a spinner at 1,200 *g* for 5 min at room temperature.

Images were obtained through use of a ScanArray Life confocal laser scanner (Perkin Elmer). Fluorescence signal intensities were collected and normalized through use of Genepix ver. 5.1 software with R language (www.r-project.org/). Differentially expressed genes were identified according to the M value ($Log_2(R/G)$), and twofold ($|M| \geq 1$) was defined as significant upregulation or downregulation. GO functional enrichment analysis (www.geneontology.org/) provided GO terms significantly enriched for differentially expressed genes as compared with the genome (Ye et al. 2006). GO enrichment analysis involved the use of EasyGo (http://bioinformatics.cau.edu.cn/easygo/) (Zhou and Su 2007). Expression patterns of unigenes under different stresses were analyzed by use of MeV software (MeV software (MultiExperiment Viewer 4.5) (www.tm4.org/mev/MeV_4_5)).

Real-time quantitative RT-PCR to confirm microarray results

Real-time quantitative RT-PCR was performed as previously described (Wei et al. 2010, 2011). In brief, first-strand cDNAs were synthesized through use of total RNA with the Rever-Tra Ace kit (Toyobo, Osaka, Japan). cDNA samples were used as templates in a 10 μL reaction system. Each gene underwent triplicate biological quantitative assays through use of Power SYBR Green Master Mix (Applied Biosystems) on the Stratagene Mx3000P system (Applied Biosystems). Gene-specific primers (Table S8) were designed with the use of PRIMEREXRESS (Applied Biosystems). Actin (FJ545641) for *P. tenuiflora* was selected as an internal control to normalize all data. Quantification of gene expression with treatments was done using the $2^{-\triangle\triangle(ct)}$ method (Livak and Schmittgen 2001; Whelan et al. 2003).

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410.

Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55,** 373–399.

Ardie SW, Xie L, Takahashi R, Liu S, Takano T (2009) Cloning of a high-affinity K^+ transporter gene PutHKT2;1 from *Puccinellia tenuiflora* and its functional comparison with OsHKT2;1 from rice in yeast and *Arabidopsis*. *J. Exp. Bot.* **60,** 3491–3502.

Arnon DI, Fratzke WE, Johnson CM (1942) Hydrogen ion concentration in relation to absorption of inorganic nutrients higher plants. *Plant Physiol.* **17,** 515–524.

Bhatnagar-Mathur P, Vadez V, Sharma KK (2008) Transgenic approaches for abiotic stress tolerance in plants: Retrospect and prospects. *Plant Cell Rep.* **27,** 411–424.

Bohnert HJ, Ayoubi P, Borchert C, Bressan RA, Burnap RL, Cushman JC, Cushman MA, Deyholos M, Fischer R, Galbraith DW, Hasegawac PM, Jenksc M, Kawasakia S, Koiwac H, Koreedae S, Leeb BH, Michalowskia CB, Misawad E, Nomurae M, Ozturka N, Postierd B, Praded R, Song CP, Tanaka Y, Wang H, Zhu JK (2001) A genomics approach towards salt stress tolerance. *Plant Physiol. Bioch.* **39,** 295–311.

Boudsocq M, Lauriere C (2005) Osmotic signaling in plants: Multiple pathways mediated by emerging kinase families. *Plant Physiol.* **138,** 1185–1194.

Campbell SA, Nishio JN (2000) Iron deficiency studies of sugar beet using an improved sodium bicarbonate-buffered hydroponic growth system. *J. Plant Nutr.* **23,** 741–757.

Caracuel Z, Casanova C, Roncero MI, Di Pietro A, Ramos J (2003) pH response transcription factor PacC controls salt stress tolerance and expression of the P-Type Na^+-ATPase Ena1 in *Fusarium oxysporum*. *Eukaryot. Cell* **2,** 1246–1252.

Century K, Reuber TL, Ratcliffe OJ (2008) Regulating the regulators: The future prospects for transcription-factor-based agricultural biotechnology products. *Plant Physiol.* **147,** 20–29.

Chinnusamy V, Zhu J, Zhu JK (2006) Salt stress signaling and mechanisms of plant salt tolerance. *Genet. Eng. (N Y)* **27,** 141–177.

Degenhardt B, Gimmler H, Hose E, Hartung W (2000) Effect of alkaline and saline substrates on ABA contents, distribution and transport in plant roots. *Plant Soil* **225,** 83–94.

ElSamad HMA, Shaddad MAK (1996) Comparative effect of sodium carbonate, sodium sulphate, and sodium chloride on the growth and related metabolic activities of pea plants. *J. Plant Nutr.* **19,** 717–728.

Ghars MA, Parre E, Debez A, Bordenave M, Richard L, Leport L, Bouchereau A, Savoure A, Abdelly C (2008) Comparative salt tolerance analysis between *Arabidopsis thaliana* and *Thellungiella halophila*, with special emphasis on K(+)/Na(+) selectivity and proline accumulation. *J. Plant Physiol.* **165,** 588–599.

Gong Q, Li P, Ma S, Indu Rupassara S, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. *Plant J.* **44,** 826–839.

Greenway H, Munns R (1980) Mechanisms of salt tolerance in non-halophytes. *Annu. Rev. Plant Phys.* **31,** 149–190.

Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H (1996) Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci. USA* **93,** 11274–11279.

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* **13,** 889–905.

Kiegerl S, Cardinale F, Siligan C, Gross A, Baudouin E, Liwosz A, Eklof S, Till S, Bogre L, Hirt H, Meskiene I (2000) SIMKK, a mitogen-activated protein kinase (MAPK) kinase, is a specific activator of the salt stress-induced MAPK, SIMK. *Plant Cell* **12,** 2247–2258.

Kobayashi S, Abe N, Yoshida KT, Liu S, Takano T (2012) Molecular cloning and characterization of plasma membrane- and vacuolar-type Na(+)/H(+) antiporters of an alkaline-salt-tolerant monocot, *Puccinellia tenuiflora*. *J. Plant Res.* **125,** 587–594.

Kodama Y, Tamura T, Hirasawa W, Nakamura K, Sano H (2009) A novel protein phosphorylation pathway involved in osmotic-stress response in tobacco plants. *Biochimie* **91,** 533–539.

Lager I, Andreasson O, Dunbar TL, Andreasson E, Escobar MA, Rasmusson AG (2010) Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor responses. *Plant Cell Environ.* **33,** 1513–1528.

Lin H, Yang Y, Quan R, Mendoza I, Wu Y, Du W, Zhao S, Schumaker KS, Pardo JM, Guo Y (2009) Phosphorylation of SOS3-LIKE CALCIUM BINDING PROTEIN8 by SOS2 protein kinase stabilizes their protein complex and regulates salt tolerance in *Arabidopsis*. *Plant Cell* **21,** 1607–1619.

Liu H, Zhang X, Takano T, Liu S (2009) Characterization of a Put CAX1 gene from *Puccinellia tenuiflora* that confers Ca^{2+} and Ba^{2+} tolerance in yeast. *Biochem. Biophys. Res. Commun.* **383,** 392–396.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25,** 402–408.

Logan BA, Kornyeyev D, Hardison J, Holaday AS (2006) The role of antioxidant enzymes in photoprotection. *Photosynth. Res.* **88,** 119–132.

Meskiene I, Baudouin E, Schweighofer A, Liwosz A, Jonak C, Rodriguez PL, Jelinek H, Hirt H (2003) Stress-induced protein phosphatase 2C is a negative regulator of a mitogen-activated protein kinase. *J. Biol. Chem.* **278,** 18945–18952.

Mukhopadhyay A, Vij S, Tyagi AK (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc. Natl. Acad. Sci. USA* **101,** 6309–6314.

Munns R, Tester M (2008) Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* **59,** 651–681.

Pang Q, Chen S, Dai S, Chen Y, Wang Y, Yan X (2010) Comparative proteomics of salt tolerance in *Arabidopsis thaliana* and *Thellungiella halophila*. *J. Proteome Res.* **9,** 2584–2599.

Peng YL, Gao ZW, Gao Y, Liu GF, Sheng LX, Wang DL (2008) Eco-physiological characteristics of alfalfa seedlings in response to various mixed salt-alkaline stresses. *J. Integr. Plant Biol.* **50,** 29–39.

Peng YH, Zhu YF, Mao YQ, Wang SM, Su WA, Tang ZC (2004) Alkali grass resists salt stress through high [K+] and an endodermis barrier to Na^+. *J. Exp. Bot.* **55,** 939–949.

Sahi C, Singh A, Kumar K, Blumwald E, Grover A (2006) Salt stress response in rice: Genetics, molecular biology, and comparative genomics. *Funct. Integr. Genomic.* **6,** 263–284.

Santos AP, Serra T, Figueiredo DD, Barros P, Lourenco T, Chander S, Oliveira MM, Saibo NJ (2011) Transcription regulation of abiotic stress responses in rice: A combined action of transcription factors and epigenetic mechanisms. *OMICS* **15,** 839–857.

Shen P, Wang R, Jing W, Zhang W (2011) Rice phospholipase Dα is involved in salt tolerance by the mediation of H^+-ATPase activity and transcription. *J. Integr. Plant Biol.* **53,** 289–299.

Shi DC, Yin SJ, Yang GH, Zhao KF (2002) Citric acid accumulation in an alkali-tolerant plant *Puccinellia tenuiflora* under alkaline stress. *Acta Bot. Sinica* **44,** 537–540.

Shiu SH, Bleecker AB (2003) Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis*. *Plant Physiol.* **132,** 530–543.

Sun G, Peng Y, Hongbo S, Liye C, Xining Z, Haiyan M, Wenzhong C, Cunxu W (2005) Does *Puccinelia tenuiflora* have the ability of salt exudation? *Colloids Surf. B. Biointerfaces* **46,** 197–203.

Tang C, Robson AD, Longnecker NE, Greenway H (1993) Physiological responses of lupin roots to high pH. *Plant Soil* **155,** 509–512.

Tatusov RL, Galperin MY, Natale DA, Koonin EV (2000) The COG database: A tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* **28,** 33–36.

Tatusov RL, Natale DA, Garkavtsev IV, Tatusova TA, Shankavaram UT, Rao BS, Kiryutin B, Galperin MY, Fedorova ND, Koonin EV (2001) The COG database: New developments in phylogenetic classification of proteins from complete genomes. *Nucleic Acids Res.* **29,** 22–28.

Wan CG, Zou XY (1990) A study on salt tolerance of *Puccinellia chinampoensis* and its' desalinizing effect on the soil. *Pratacultural Sci.* **3**.

Wang C, Li J, Yuan M (2007a) Salt tolerance requires cortical microtubule reorganization in *Arabidopsis*. *Plant Cell Physiol.* **48,** 1534–1547.

Wang C, Zhang L, Yuan M, Ge Y, Liu Y, Fan J, Ruan Y, Cui Z, Tong S, Zhang S (2010a) The microfilament cytoskeleton plays a vital role in salt and osmotic stress tolerance in *Arabidopsis*. *Plant Biol.* (*Stuttg*) **12,** 70–78.

Wang C, Zhang LJ, Huang RD (2011) Cytoskeleton and plant salt stress tolerance. *Plant Signal. Behav.* **6,** 29–31.

Wang CM, Zhang JL, Liu XS, Li Z, Wu GQ, Cai JY, Flowers TJ, Wang SM (2009) *Puccinellia tenuiflora* maintains a low Na^+ level under salinity by limiting unidirectional Na^+ influx resulting in a high selectivity for K^+ over Na^+. *Plant Cell Environ.* **32,** 486–496.

Wang JY, Xia XL, Wang JP, Yin WL (2008) Stress responsive zinc-finger protein gene of *Populus euphratica* in tobacco enhances salt tolerance. *J. Integr. Plant Biol*. **50,** 56–61.

Wang S, Bai Y, Shen C, Wu Y, Zhang S, Jiang D, Guilfoyle TJ, Chen M, Qi Y (2010b) Auxin-related gene families in abiotic stress response in Sorghum bicolor. *Funct. Integr. Genomic.* **10,** 533–546.

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta* **218,** 1–14.

Wang Y, Chu Y, Liu G, Wang MH, Jiang J, Hou Y, Qu G, Yang C (2007b) Identification of expressed sequence tags in an alkali grass (*Puccinellia tenuiflora*) cDNA library. *J. Plant Physiol.* **164,** 78–89.

Wang Y, Yang C, Liu G, Jiang J (2007c) Development of a cDNA microarray to identify gene expression of *Puccinellia tenuiflora* under saline-alkali stress. *Plant Physiol. Biochem.* **45,** 567–576.

Wei LQ, Xu WY, Deng ZY, Su Z, Xue Y, Wang T (2010) Genome-scale analysis and comparison of gene expression profiles in developing and germinated pollen in *Oryza sativa*. *BMC Genomics* **11,** 338.

Wei LQ, Yan LF, Wang T (2011) Deep sequencing on genome-wide scale reveals the unique composition and expression patterns of microRNAs in developing pollen of *Oryza sativa*. *Genome Biol.* **12,** R53.

Whelan JA, Russell NB, Whelan MA (2003) A method for the absolute quantification of cDNA using real-time PCR. *J. Immunol. Methods* **278,** 261–269.

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* **6,** 251–264.

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.* **57,** 781–803.

Yan X, Sun G, Li J, Xiao W (1999) Changes of several osmotica in *Puccinellia tenuiflora* seedling under alkali salt stress. *Bull. Bot. Res.* **19,** 347–355.

Yang CW, Xu HH, Wang LL, Liu J, Shi DC, Wang DL (2009) Comparative effects of salt-stress and alkali-stress on the growth, photosynthesis, solute accumulation, and ion balance of barley plants. *Photosynthetica* **47,** 79–86.

Ye J, Fang L, Zheng H, Zhang Y, Chen J, Zhang Z, Wang J, Li S, Li R, Bolund L (2006) WEGO: A web tool for plotting GO annotations. *Nucleic Acids Res.* **34,** W293–W297.

Yu J, Chen S, Zhao Q, Wang T, Yang C, Diaz C, Sun G, Dai S (2011) Physiological and proteomic analysis of salinity tolerance in *Puccinellia tenuiflora*. *J. Proteome Res.* **10,** 3852–3870.

Zhang C, Shunsaku N, Shenkui L, Tetsuo T (2008) Characterization of two plasma membrane protein 3 genes (PutPMP3) from the alkali grass, *Puccinellia tenuiflora*, and functional comparison of the rice homologues, OsLti6a/b from rice. *BMB Rep.* **41,** 448–454.

Zhang H, Han B, Wang T, Chen S, Li H, Zhang Y, Dai S (2012) Mechanisms of plant salt response: Insights from proteomics. *J. Proteome Res.* **11,** 49–67.

Zhong NQ, Han LB, Wu XM, Wang LL, Wang F, Ma YH, Xia GX (2012) Ectopic expression of a bacterium NhaD-type Na^+/H^+ antiporter leads to increased tolerance to combined salt/alkali stresses. *J. Integr. Plant Biol.* **54,** 412–421.

Zhou X, Su Z (2007) EasyGO: Gene Ontology-based annotation and functional enrichment analysis tool for agronomical species. *BMC Genomics* **8,** 246.

Zhou XF, Wang XZ (2002) Research progress in salt tolerance genes of SOS in *Arabidopsis thaliana*. *Yi Chuan* **24,** 190–192.

Zhu JK (2000) Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiol.* **124,** 941–948.

Zhu JK (2001a) Cell signaling under salt, water and cold stresses. *Curr. Opin. Plant Biol.* **4,** 401–406.

Zhu JK (2001b) Plant salt tolerance. *Trends Plant Sci.* **6,** 66–71.

Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* **53,** 247–273.

Zhu JK, Liu J, Xiong L (1998) Genetic analysis of salt tolerance in *Arabidopsis*: Evidence for a critical role of potassium nutrition. *Plant Cell* **10,** 1181–1191.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Water content of *Puccinellia tenuiflora* under various stresses.

Table S2. Annotation of 4,982 unigenes generated by expressed sequence tag sequencing.

Table S3. Internal standard genes spotted in microarray.

Table S4. Fluorescence values of unigenes on microarray assay.

Table S5. M values for 1,105 differentially expressed genes.

Table S6. Functional categories of differentially expressed genes.

Table S7. Gene ontology annotation of differentially expressed genes.

Table S8. Validation by quantitative reverse transcription polymerase chain reaction.