



RESEARCH PAPER

Identification of early salt stress response genes in tomato root by suppression subtractive hybridization and microarray analysis

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Abstract

High salinity is one of the most serious threats to crop production. To understand the molecular basis of plant responses to salt stress better, suppression subtractive hybridization (SSH) and microarray approaches were combined to identify the potential important or novel genes involved in the early stage of tomato responses to severe salt stress. First, SSH libraries were constructed for the root tissue of two cultivated tomato (*Solanum lycopersicum*) genotypes: LA2711, a salt-tolerant cultivar, and ZS-5, a salt-sensitive cultivar, to compare salt treatment and non-treatment plants. Then a subset of clones from these SSH libraries were used to construct a tomato cDNA array and microarray analysis was carried out to verify the expression changes of this set of clones upon a high concentration of salt treatment at various time points compared to the corresponding non-treatment controls. A total of 201 non-redundant genes that were differentially expressed upon 30 min of severe salt stress either in LA2711 or ZS-5 were identified from microarray analysis; most of these genes have not previously been reported to be associated with salt stress. The diversity of the putative functions of these genes indicated that salt stress resulted in a complex response in tomato plants.

Key words: Gene expression, genotype, microarray, salt stress, SSH, tomato.

Introduction

Abiotic stress, such as drought, high salinity, extreme temperature, and flooding is a major cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray *et al.*, 2000). In particular, salinity is becoming an increasingly global problem and affects approximately 20% of global irrigated agricultural land (Flowers and Yeo, 1995). In China, around 100 million hectares of land, distributed over 16 provinces, have been affected by increased salinity (Zhao *et al.*, 2002). The situation is becoming even worse due to the boom in protected agriculture, especially with regard to vegetables grown in such facilities. Currently there are over 1.5 million hectares of protected vegetables in China for which secondary salinization is an ever-present threat to the yield and quality of vegetables. Almost all major vegetable crops, for example, pepper, eggplant, potato, lettuce, and cabbage, are susceptible to increased salinity (Shannon and Grieve, 1999).

Plant salt tolerance is a complex trait controlled by multiple genes. Considerable attention has been directed toward elucidating the molecular basis of plant salt

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tolerance during recent years and numerous salt tolerance-related genes have been identified. Major studies on plant salt tolerance have been focused on *Arabidopsis*. As a consequence, several important pathways involved in salt stress signal transduction have been identified, although some key components in these pathways still remain to be defined (Xiong *et al.*, 2002). These pathways include Salt Over Sensitive (SOS) pathway (SOS3-SOS2-SOS1) which regulates ion homeostasis under salt stress and results in Na⁺ efflux and vacuolar compartmentation (Sanchez-Barrena *et al.*, 2005); the Calcium Dependent Protein Kinase (CDPK) pathway, which plays an important role in osmotic stress (Sanchez-Barrena *et al.*, 2005); and the Mitogen Activated Protein (MAP) kinase pathway, which is important for counteracting both abiotic and biotic stresses (Nakagami *et al.*, 2005). In addition, two plant hormones, abscisic acid (ABA) and ethylene, also play an important role in the complicated story of abiotic stress and, consequently, cross-talk between these two hormones has been reported (Tanaka *et al.*, 2005). Numerous reports have shown that different pathways are interconnected and co-ordinately regulate the plant response to biotic and abiotic stresses (Ludwig *et al.*, 2005; Ma *et al.*, 2006).

Tomato is a member of the family Solanaceae that includes several additional economically important crops such as potato, pepper, and eggplant, and as such, represents the most valuable plant family in terms of vegetable crops. Compared with *Arabidopsis*, much less is known about the mechanism behind tomato plant responses to salt stress, although significant progress has been made in this regard during recent years (Cuartero *et al.*, 2006). Nearly one dozen QTLs conferring salt tolerance have been identified in tomato through extensive QTL mapping (Foolad *et al.*, 2001). Wei *et al.* (2000) identified 20 cDNAs which are responsive to salt treatment using differential display PCR. Huang *et al.* (2004) identified a novel ethylene responsive factor called TERF1 from tomato, which might function as a link between the ethylene and osmotic stress pathways. Transgenic tobacco plants overexpressing this gene exhibited increased salt tolerance. By analysing tomato salt-hypersensitive (*ts*) mutants, Borsani *et al.* (2001) were able to identify two loci, the *TSS1* and *TSS2*; of which the *TSS1* locus is essential for potassium nutrition and salt tolerance while *TSS2* plays an important role in the interactions between salt tolerance and abscisic acid signalling.

Despite such advances, much remains to be elucidated about the molecular basis of plant responses to salt stress. To help decipher the complex molecular mechanisms of plant salt tolerance, a strategy of combining suppression subtractive hybridization (SSH) and cDNA microarray technologies was used to identify salt stress-regulated genes on a large scale from two cultivated tomato genotypes: LA2711, a salt-tolerant cultivar, and ZS-5, a salt-sensitive cultivar. The analysis focused on early response genes

after severe salt stress in the root tissue of tomato seedlings. Due to its role in absorbing water and nutrients, the root is the foremost part of the plant to encounter soil salinity. Upon salt stress, the expression of certain genes in the root was changed within 15 min (Kawasaki *et al.*, 2001). These genes are usually designated as early response genes which are probably the stress sensors or inducible transcriptional activators or upstream signal pathway components and thus may act as the fate dominators of salt tolerance. In this study, a set of salt stress response genes was identified that had not previously been reported to be associated with salt stress.

Materials and methods

Plant materials and screening of tomato salt tolerance

Seeds of LA2711, a salt-tolerant cultivar, and ZS-5, a sensitive cultivar, were used for germination test. For each cultivar 120 hand-selected seeds of uniform size were surface-sterilized in 70% (v/v) ethanol for 15 s, followed by 4% (w/v) sodium hypochlorite for 15 min, then rinsed several times with sterile distilled water. The seeds, 30 per Petri dish, were then sown on MS medium (Murashige and Skoog, 1962) containing 30 g l⁻¹ sucrose and 7 g l⁻¹ agar, supplemented with 100 mM NaCl for salt treatment or nothing for control. Four replicates were used in each treatment. Seeds were allowed to germinate at approximately 24 °C in the dark for 48 h and were then transferred to an incubator with a 16 h photoperiod and an optimal temperature regime of 24/21 °C (light/dark). The germination response was scored visually as radicle protrusion at 12 h intervals for 27 consecutive days.

To investigate the physiological changes of LA2711 and ZS-5 upon salt stress, the level of selected ions and enzymes was measured at the seedling stage. Seeds for both genotypes were germinated in a tissue culture room and grown hydroponically in black plastic pots containing 1.8 l of modified one-fifth Johnson's solution supplemented with 10 µM Fe-EDDHA (Wang *et al.*, 2001). Only one seedling was grown in each pot in a greenhouse with modest aeration provided.

After 5 weeks of growth, plants were transferred either to a new nutrient solution with 150 mM NaCl for salt stress treatment or to a nutrient solution without salt as the control. Net photosynthetic rate per unit area, stomatal conductance, and evaporation rate of the third leaves from the top were measured after 7 d treatment using a TPS-1 photosynthesis system (PP System, UK). All measurements were made at a photosynthetic photon flux (400–700 nm) of 200–250 mmol m⁻² s⁻¹. Eight days after salt treatment, the youngest fully expanded leaves and root tips were sampled for the determination of ions. The leaf and root Na⁺ and K⁺ concentrations were determined by flame photometry as described in Asch *et al.* (2000).

Stress treatment

Plants of LA2711 and ZS-5 were grown hydroponically as described above. The nutrient solution was refreshed after 28 d and 35 d of growth. Three days after the second refreshment of nutrient solution, the seedlings were transferred either into a new nutrient solution with 300 mM NaCl for salt stress treatment or a nutrient solution without salt as the control.

Total RNA and mRNA isolation

Roots were harvested separately at 15, 30 min, 1, 2, 6, 12, and 24 h after the treatment, frozen in liquid nitrogen, and then kept at

–70 °C. To minimize the effects due to light/dark exposure and/or circadian regulated responses, all samples except for the one at 12 h were harvested during periods of light exposure.

Frozen root samples were ground in liquid nitrogen. Total RNA was isolated using TriZOL reagent (GIBCOL/BRL) according to the manufacturer's instructions. For suppression subtracted hybridization, equal amounts of total RNA for each sample from treatment or control were mixed and the mRNA was purified from the mixed total RNA using the Dynabeads mRNA Purification Kit (DynaL Biotech) according to the manufacturer's protocol. The quantity and quality of isolated total RNA was examined by spectrophotometry and gel electrophoresis, respectively.

Construction of subtracted cDNA library

The cDNA reversely transcribed from 2 µg of the mixed mRNA mentioned above was used for suppressive subtractive hybridization with the Clontech PCR Select-cDNA Subtraction Kit (BD Biosciences Clontech). Both forward (salt treatment as tester and non-salt-treatment control as driver) and reverse (non-salt-treatment control as tester and salt treatment as driver) SSH cDNA libraries were constructed for LA2711 and ZS-5, respectively, following the manufacturer's instruction with slight modifications.

In brief, driver and tester cDNAs were digested with *Rsa*I, phenol/chloroform-extracted, ethanol-precipitated, and resuspended in water. Tester cDNA was split into two pools and then ligated to a different adaptor (supplied in the cDNA subtraction kit). Two rounds of hybridization and PCR amplification were carried out to normalize and enrich the differentially expressed cDNAs according to the manufacturer's protocol with the following changes: the primary PCR was performed for 27 cycles (94 °C, 15 s; 66 °C, 30 s; 72 °C, 90 s), the secondary PCR was performed for 13 cycles (94 °C, 30 s; 68 °C, 30 s; 72 °C, 90 s) and the secondary PCR products were purified and inserted into pGEM T-easy Vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5 α competent cells.

Amplification of cDNA inserts

Seven hundred and sixty eight cDNA clones were randomly selected from each of the four SSH libraries. The clones, freshly grown overnight at 37 °C, were used as the templates. The cDNA inserts were amplified by PTC-100 programmable Thermal Controllers (MJ Research, Waltham, MA, USA) using nested PCR primers 1 and 2R provided in the PCR Select-cDNA Subtraction Kit, which were complementary to sequences flanking both ends of the cDNA insert. The 100 µl amplification reaction mixtures contained 68.7 µl sterile water, 10 µl 10 \times buffer, 0.75 µl of each primer (50 µM each), 2 µl dNTPs (10 mM each), 6 µl MgCl₂ (25 mM), 10 µl 50% (v/v) glycerol, 4 units of *Taq* DNA polymerase (MBI Ferments, Hanover, MD, USA), and 1 µl of bacterial culture. Thermocycling conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, 60 °C for 30 s, and 72 °C for 2.5 min, then a final extension at 72 °C for 5 min and held at 4 °C. PCR products were precipitated with anhydrous ethanol, resuspended in 40 µl sterile water, and run on 0.8% agarose gel and examined by Beckman DU520 UV Spectroscopy to ensure the quality and quantity, and stored in 384-well microtitre plates.

cDNA microarray slides preparation

The PCR products were precipitated one more time with the addition of 100 µl of anhydrous ethanol and resuspended in 15 µl of 50% dimethylsulphoxide (DMSO) at a final concentration of 0.1–0.5 µg µl⁻¹ and then spotted onto amino silanated glass slides (CapitalBio. Corp, Beijing, China) with a SmartArrayerTM micro-

arrayer (CapitalBio Corp.). Each clone was printed in triplicate. After printing, the slides were baked for 1 h at 80 °C and stored dry at room temperature till use. Prior to hybridization, the slides were rehydrated over 65 °C water for 10 s, snap dried on a 100 °C heating block for 5 s, and UV cross-linked at 250 mJ cm⁻². The unimmobilized PCR products were washed off with 0.5% SDS for 15 min at room temperature and SDS was removed by dipping the slides in anhydrous ethanol for 30 s. The slides were spin-dried at 1000 rpm for 2 min.

Eight sequences derived from intergenic regions in yeast genome, showing no significant homology to all the existing sequences in GenBank, were spotted multiple times onto the microarray as exogenous controls. Total tomato RNA was spiked with a mixture of these exogenous control RNAs to validate the semi-quantitative microarray result.

Preparation of fluorescent dye-labelled cDNA and hybridization

The gene expression profiles in root tissue after 30 min, 2 h, and 6 h severe salt stress and the corresponding non-stressed controls were investigated by microarray analysis. An aliquot of 5 µg total RNA was used to produce Cy5/Cy3-labelled cDNA employing an RNA amplification combined with Klenow enzyme labelling strategy according to a previous published protocol (Guo *et al.*, 2005b).

Cy5/Cy3-labelled cDNA were hybridized with the microarray at 42 °C overnight. Each hybridization was performed in duplicate by dye swap. Following hybridization, the arrays were washed with 0.2% SDS, 2 \times SSC at 42 °C for 5 min, and 0.2% SSC for 5 min at room temperature.

Microarray data analysis

Arrays were scanned with a confocal laser scanner, LuxScanTM 10K (CapitalBio Corp.), and the resulting images were analysed with SpotData Pro 2.0 software (CapitalBio Corp.). Spots with fewer than 50% of the signal pixels exceeding the local background value for both channels (Cy3 and Cy5) plus two standard deviations of the local background were removed. This step further ensured that spots with characteristic doughnut shapes, often encountered on microarrays, would not be part of the subsequent analysis. A spatial and intensity-dependent (LOWESS) normalization method was employed to normalize the ratio values (Yang *et al.*, 2002). Normalized ratio data were then log transformed. cDNA spots with less than four out of total six data points in each replicated hybridization were removed. Differentially expressed genes were identified using *t* test and multiple test corrections were performed using False Discovery Rate (FDR, Benjamini and Hochberg, 1995). Genes with FDR <0.01 and a fold change greater than or equal to two were identified as differentially expressed genes. The microarray data and the related experiment information from this work were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE4961.

EST sequence analysis

All the cDNAs differentially expressed upon 30 min salt treatment either in LA2711 or ZS-5 were single-pass sequenced (AuGCT Biotechnology Co. Ltd, Beijing, China). The raw sequences of these ESTs, along with another set of approximately 200 ESTs on the array sequenced previously in our laboratory, were base called using PHRED (Ewing and Green, 1998; Ewing *et al.*, 1998). Low quality regions, vector and adaptor sequences were removed using LUCY program (Chou and Holmes, 2001). The sequences were also screened against UniVec database (<http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html>) and *E. coli* whole genome sequence to remove vector and *E. coli* genome contaminations. The resulting

high quality sequences were then clustered and assembled along with the TIGR tomato gene index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato) using TGICL program (Pertea et al., 2003). In total, 201 unigenes were obtained which contain ESTs differentially expressed after 30 min salt treatment either in LA2711 or ZS-5. These unigenes were then annotated by performing sequence similarity searches against the NCBI nr database using the BLASTX program with a cutoff e-value of $1e-5$. The functional classification of these unigenes was carried out based on the MIPS functional catalogue (<http://mips.gsf.de/projects/funcat>). All the EST sequences generated under this study were deposited into GenBank with accession numbers from DY523325 to DY523920.

RT-PCR analysis

Total root RNA isolated by TriZOL reagent (Invitrogen, USA) from three stages (30 min, 2 h, and 6 h) of both stressed and control plants were used for RT-PCR analysis. First strand cDNA was synthesized from 8 μ g total RNA from each sample using MMLV reverse transcriptase (Toyobo, Osaka, Japan) according to the supplier's manual. Tomato elongation factor 1 α was used as the inner control for RT-PCR analysis. All primers for the candidate genes and elongation factor 1 α were designed by the Primer3 program (<http://redb.ncpgr.cn/modules/redbtools/primer3.php>) and are shown in Supplementary Table S1 at *JXB* online. General PCR was conducted with the following program: an initial denaturation at 94 °C for 2 min, followed by 25–30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, a final extension at 72 °C for 6 min and held at 4 °C. RT-PCR experiments were repeated three times and the PCR products were detected by 1% agarose gel in 1 \times TAE with EtBr.

Results

Phenotypic performance of LA2711 and ZS-5 under salt stress

LA2711 was recorded as a salt-tolerant accession in the Tomato Genetic Research Center (TGRC, <http://tgrc.ucdavis.edu>); however, little is known about its genetic background. In this study, its performance and response under salt stress were investigated, together with ZS-5, a salt-sensitive genotype.

Under non-stress conditions LA2711 germinated faster than ZS-5. When salt was applied at a concentration of 100 mM, both genotypes exhibited delayed germination; however, LA2711 germinated much faster than ZS-5 by reaching 50% germination rate 12 d earlier (Fig. 1). The final germination rate (91.1%) of LA2711 was also significantly higher than that of ZS-5 (65.6%). These results supported the conclusion that LA2711 is a salt-tolerant genotype.

The performance of LA2711 in 150 mM salt solution during its vegetative stage was also investigated. A series of physiological indices of genotypes LA2711 and ZS-5 were evaluated at the same growth stage as that used for expression analysis (Table 1). The Na⁺ and K⁺ concentrations of leaf and root were determined under control and stressed conditions. Both genotypes exhibited an increase in Na⁺ concentration in leaf and root under salinity stress. The root Na⁺ concentration in LA2711 has

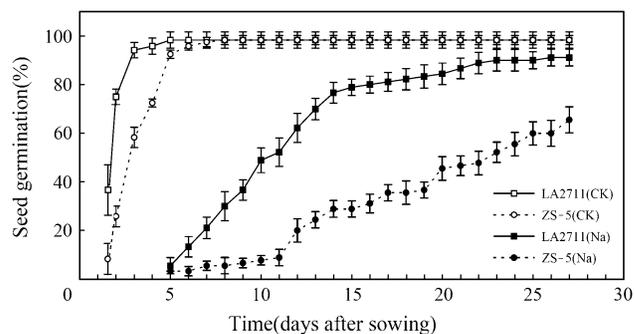


Fig. 1. Characterization of seed germination of a tomato salt-tolerant genotype, LA2711, and a salt-sensitive genotype, ZS-5, treated with 100 mM NaCl solution (Na) and in normal condition (CK). Bars are mean \pm SD of four repeats. Seeds were germinated in a Petri dish with BM media (MS medium with 30 g l⁻¹ sucrose and 7 g l⁻¹ agar) supplemented with or without 100 mM NaCl solution under optimal conditions in culture room.

no significant difference from that in ZS-5 after salt stress. However, LA2711 contained a significantly lower level of leaf Na⁺ compared to ZS-5. The leaf K⁺ level increased in LA2711 while it decreased in ZS-5 under salt stress. In addition, the leaf Na⁺/root Na⁺ ratio and leaf Na⁺/K⁺ ratio were also more favourable in the genotype LA2711.

Net photosynthetic rate per unit area, stomatal conductance, and transpiration rate were decreased markedly in response to salt stress in both genotypes. However, they were much higher in LA2711 than in ZS-5 (Table 1).

When a high concentration of salt solution (300 mM) was applied to 5-week seedlings of LA2711 and ZS-5 under the hydroponics system, seedlings of both genotypes were severely wilted within 30 min. However, LA2711 recovered sooner than ZS-5 (see Supplementary Fig. S1 at *JXB* online). In addition, analysis of the physiological (photosynthesis) and biochemical (K⁺, Ca²⁺, Mg²⁺, and Na⁺; Pro and MDA) traits of these two cultivars indicated that LA2711 performed better than ZS-5 under severe salt stress conditions (data not shown). All these results supported that LA2711 is salt-tolerant at its vegetative stage.

SSH libraries construction and overall features of the salt stress-responsive expression profile

Forward and reverse subtractions were conducted between root tissues from salt-stressed and non-stressed LA2711 and ZS-5 plants, respectively. Seven hundred and sixty eight clones were randomly picked from each SSH library. The average insert size of the SSH clones was around 0.55 kb. The clones from the four SSH libraries were amplified and used for microarray analysis. RNA samples from the root tissues at the stages of 30 min, 2 h and 6 h after salt stress and the same time points of non-treated control plants were used for microarray hybridization.

In total, 2447 differentially expressed cDNA clones (FDR <0.01 and fold change ≥ 2) from either LA2711 or

Table 1. Phenotypic responses of tomato salt-tolerant genotype, LA2711 and salt-sensitive genotype, ZS-5 under salt stress

Indicator	Treatment			
Ions ^a		Leaf Na ⁺ (mg/100 mg DW) ^c	Leaf K ⁺ (mg/100 mg DW)	Root Na ⁺ (mg/100 mg DW)
	LA2711 control	0.60±0.01	10.59±0.44	1.98±0.02
	LA2711 salt	1.77±0.23	12.42±0.47	7.88±0.16
	ZS-5 control	0.58±0.10	10.53±0.42	1.99±0.09
	ZS-5 salt	2.65±0.35	8.35±0.90	7.51±0.21
		Leaf Na ⁺ /K ⁺ ratio	Leaf Na ⁺ /root Na ⁺ ratio	
	LA2711 control	0.06	0.30	
	LA2711 salt	0.14	0.22	
	ZS-5 control	0.06	0.29	
	ZS-5 salt	0.32	0.35	
Photosynthetic ^b		Photosynthesis (μmol CO ₂ m ⁻² s ⁻¹)	Conductance (mmol m ⁻² s ⁻¹)	Evaporation rate (mmol m ⁻² s ⁻¹)
	LA2711 control	9.25±0.21	234.0±2.83	3.99±0.01
	LA2711 salt	3.50±0.07	21.0±1.41	0.60±0.04
	ZS-5 control	9.35±0.42	370.0±41.01	4.92±0.16
	ZS-5 salt	2.65±0.01	9.5±0.71	0.27±0.03

^a Data are shown as the mean ± standard error of triplicate samples.

^b Data are shown as the mean ± standard error of six replicate samples.

^c DW, dry weight.

ZS-5 were identified upon severe salt stress. Overall, the salt-sensitive and -tolerant genotypes showed very similar expression patterns. The longer the salt treatment, the more the clones changed in both genotypes, while the sensitive genotype was characterized by the up- or down-regulation of a relative larger number of clones (Fig. 2). This result agreed with a recent report on rice (Walia *et al.*, 2005).

Detailed analysis of the differentially expressed clones revealed that 92.7% of the up-regulated clones for LA2711 were from the two forward SSH libraries, and 82.5% down-regulated clones were from the two reverse SSH libraries. The corresponding ratios are 91.8% and 82.5% for the sensitive genotype, ZS-5. Upon 30 min severe salt stress, 104 up-regulated and 124 down-regulated ESTs were found in LA2711 while 177 up-regulated and 255 down-regulated ESTs were identified in ZS-5. Because early response genes are probably critical in the activation and integration of stress defence mechanisms and thus play important roles in the process of plant fighting against salt stress, interest was focused on genes which were highly regulated by 30 min severe salt stress. However, their expression changes after 2 h and 6 h severe salt stress were also investigated by microarray analysis in order to gain a better understanding of their responses to salt stress.

All the differentially expressed clones at 30 min of severe salt stress were sequenced. After removing low quality regions, vector and adaptor sequences, the remaining high quality sequences along with the TIGR tomato gene index were clustered and assembled into unigenes to remove redundancy. Two hundred and one unique genes were obtained from these ESTs; among them, 80 were up-regulated and 121 were down-regulated. In the up-regulated genes, 11 were only identified in LA2711, 43 were only identified in ZS-5, and 26 were identified in both genotypes (Fig. 2B). As for the down-regulated genes, 14

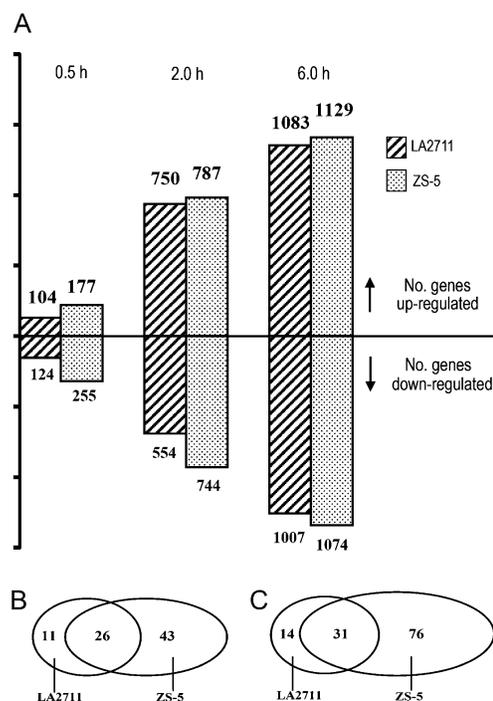


Fig. 2. Number of differentially expressed clones/genes. (A) Number of SSH cDNA clones significantly up- or down-regulated in LA2711 and ZS-5 in response to salt stress at various time points. (B, C) Venn diagram illustrates the number of unique genes up (B) or down-regulated (C) by salt stress in either or both genotypes.

were from LA2711 only, 76 were from ZS-5 only, and 31 were from both (Fig. 2C). This detailed analysis also suggested a relatively larger number of genes changed significantly in the sensitive genotype ZS-5.

All 201 differentially expressed genes were functionally annotated by blasting against the GenBank non-redundant protein database, and subsequently classified into 14

functional categories according to their putative functions (Fig. 3; see Supplementary Table S2 at *JXB* online). Genes assigned to the metabolism category accounted for the largest group in both LA2711 and ZS-5, and transcriptional genes and unknown genes represented the second largest group in LA2711 and ZS-5, respectively. In categories such as ‘transcription’ and ‘interaction with the cellular environment’, genes were mostly up-regulated for at least one time point in LA2711 and ZS-5. In categories such as ‘metabolism’ and ‘energy’, a large portion of genes were down-regulated from early time

points (30 min or 2 h) to a later time point (6 h) either in LA2711 or ZS-5.

Verification of microarray data

To validate the changes in mRNA abundance detected by microarray analysis, RNA was isolated from the roots of additional plants under severe salt stress and non-stressed as controls. RT-PCR was performed on eight differentially expressed genes, as based on the microarray analysis. RT-PCR data agreed with the microarray data for 40 out of 48 (83%) data points (Fig. 4). Due to the possible

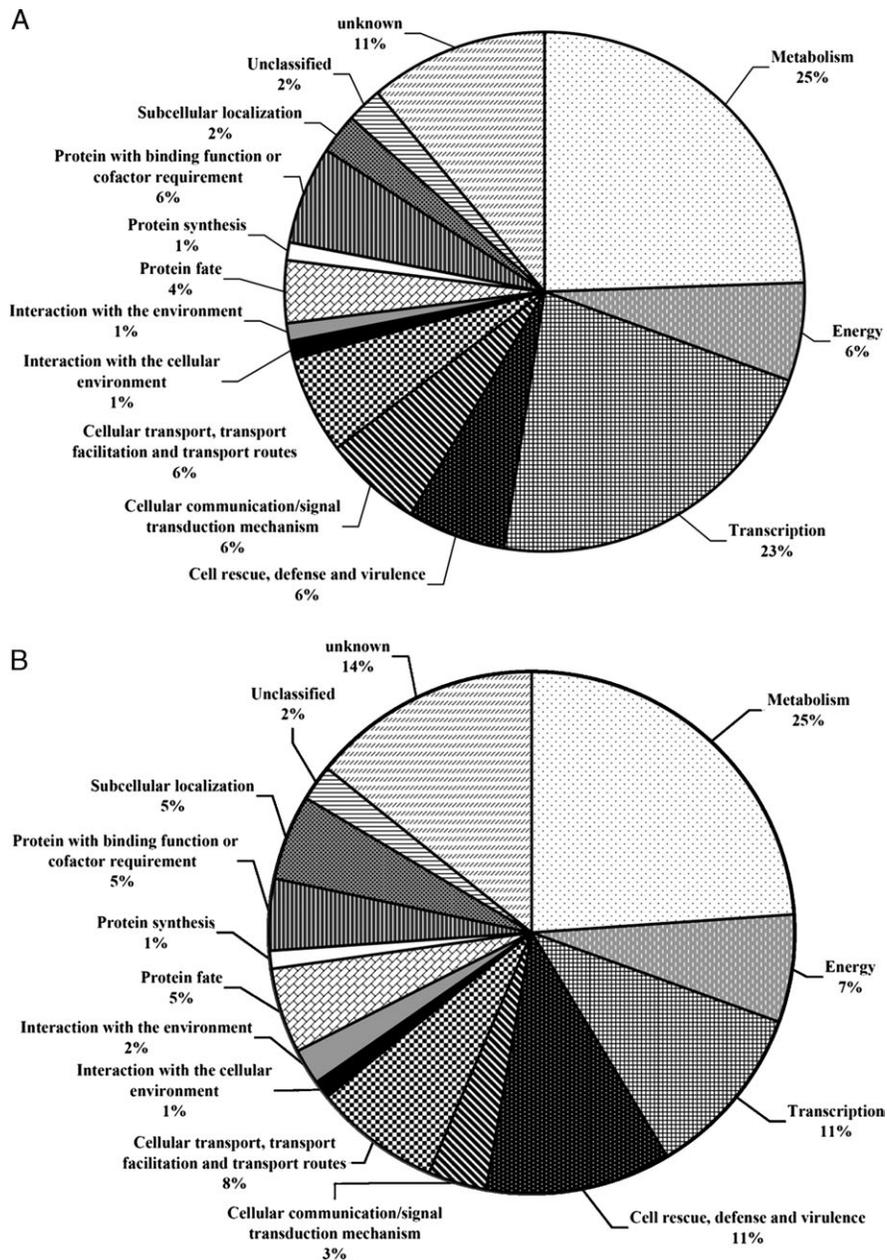
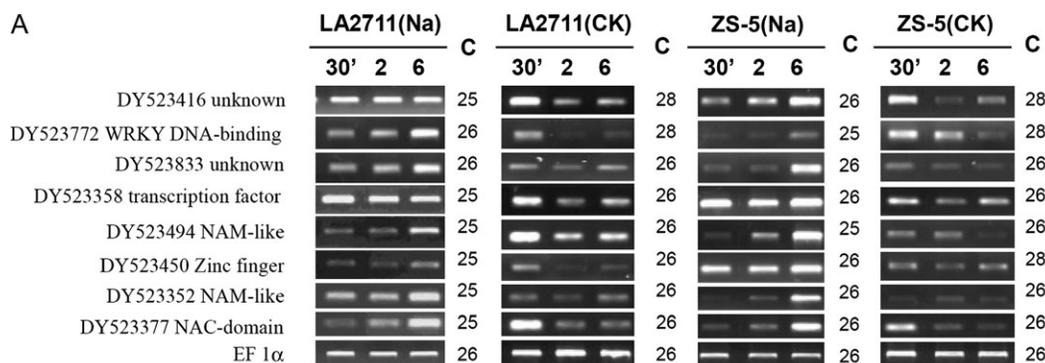


Fig. 3. Distribution of differentially expressed genes in the two genotypes respectively (A, LA2711; B, ZS-5). A total of 201 unique ESTs were grouped into 14 functional categories based on MIPS functional categories. The percentage of gene transcripts in each group is listed.

**B**

Accession No.	Annotation	e value	LA2711			ZS-5		
			0.5h	2h	6h	0.5h	2h	6h
DY523416	unknown protein	1e-157	2.18	20.5	17.25	1.5	75.4	25.92
DY523772	WRKY DNA-binding protein 4	5e-57	-2.11	11.94	16.64	-1.07	9.12	9.28
DY523833	unknown protein	0.0	-1.02	3.45	13.27	2.01	4.82	17.92
DY523358	transcription factor	1e-146	4.43	5.03	3.09	2.31	5.63	3.45
DY523494	nam-like protein 10	1e-125	2.24	13.68	13.65	2.22	14.78	14.58
DY523450	zinc finger protein PIF1	1e-121	-1.23	4.09	3.41	-2.24	9.96	3.52
DY523352	nam-like protein 10	1e-152	5.36	5.92	7.19	3.68	7.25	10.61
DY523377	NAC domain protein	1e-119	-1.86	3.07	10.02	-2.18	4.65	10.45

Fig. 4. Verification of microarray results by RT-PCR. (A) RT-PCR analysis of the selected genes. Number of PCR cycles is listed on the right side. LA2711 (Na): sample treated by 300 mM salt solution for LA2711; LA2711 (CK): samples without salt stress for LA2711; ZS-5 (Na): sample treated by 300 mM salt solution for ZS-5; LA2711 (CK): samples without salt stress for ZS-5; different time points are listed on the top; (B) Expression ratios of the selected genes from microarray analysis.

cross-hybridization of probes on cDNA microarray and other inevitable technical errors introduced either from microarray or RT-PCR, it was concluded that overall there was a good agreement between the microarray data and the RT-PCR results.

Transcription related genes are changed under severe salt stress

In response to a short period of time (30 min) of severe salt stress, 18 and 19 transcriptional genes were changed significantly in LA2711 and ZS-5, respectively, accounting for 23% of 82 early differentially expressed genes in LA2711, and 11% of 176 early differentially expressed genes in ZS-5. Among these differentially expressed transcriptional genes, genes belonging to the three major families of putative transcription factors were identified (Table 2).

The first is the NAC family which has a highly conserved domain called NAC (petunia NAM, *Arabidopsis* ATAF1 and CUC2 genes). Three NAM-like genes (DY523352, DY523417, DY523494) and a NAC gene (DY523377), which was previously reported to be involved in the interaction of tomato and virus (Selth *et al.*, 2005), were identified as salt stress-responsive genes in

this study. All the three NAM-like genes were continually induced by salt treatment. As for the NAC gene, it was inhibited at the early stage of salt exposure (30 min), and then was induced at the later stages (2 h and 6 h).

The second is the EREBP (ethylene response element binding protein) family. Six genes belonging to this family were identified. They are DY523793 (ripening regulated protein DDTFR10/A), DY523349 (ethylene-binding protein), DY523801 (AP2-domain DNA-binding protein), DY523655 (transcription factor JERF1), DY523820 (CRT/DRE binding factor 1), and DY523823 (ethylene response factor 3). In both cultivars, DY523801, DY523823, and DY523793 were continuously induced throughout the salt treatment while DY523820 was found down-regulated rapidly and then up-regulated after 2 h of salt stress. DY523349 did not change significantly in LA2711, but was repressed significantly in ZS-5 in the first 30 min; then it was dramatically induced after 2 h of salt stress in both cultivars. The expression of DY523655 did not change significantly at 30 min and 2 h, but was significantly repressed at a later time point (6 h) in LA2711. By contrast, it was down-regulated significantly at 30 min and 2 h but unchanged at 6 h of salt exposure in ZS-5.

Table 2. List of transcription factors highly regulated by severe salt stress in tomatoSignificant difference (FDR <0.01 and fold change \geq 2) in relative level is shown in bold.

Accession no.	Annotation	e-value	LA2711			ZS-5		
			0.5 h	2 h	6 h	0.5 h	2 h	6 h
DY523352	Nam-like protein 10	1e-152	5.36	5.92	7.19	3.68	7.25	10.61
DY523417	Nam-like protein 10	5e-14	2.20	12.61	23.53	2.33	22.37	18.38
DY523494	Nam-like protein 10	1e-125	2.24	13.68	13.65	2.22	14.78	14.58
DY523377	NAC domain protein	1e-119	-1.86	3.07	10.02	-2.18	4.65	10.45
DY523349	Ethylene-binding protein-like	6e-32	-1.45	26.90	12.62	-4.00	53.95	14.49
DY523655	Transcription factor JERF1	0.0	-1.49	-1.88	-2.19	-2.24	-2.54	-1.51
DY523793	Ripening regulated protein DDTFR10/A	1e-117	3.54	2.62	6.73	3.20	3.35	7.20
DY523801	AP2-domain DNA-binding protein	7e-43	3.20	25.27	20.97		27.78	32.43
DY523820	CRT/DRE binding factor 1	3e-36	-3.21	11.05	14.68	-3.14	14.13	11.94
DY523823	Ethylene response factor 3	1e-117	2.08	2.51	6.72	2.65	4.01	7.08
DY523398	Zinc-finger protein	1e-127	2.27	5.94	13.59	2.48	22.93	18.55
DY523450	Zinc-finger protein PIF1	1e-121	-1.23	4.09	3.41	-2.24	9.96	3.52
DY523481	RING-H2 subgroup RHE protein	8e-68	3.84	5.09	7.05	2.70	3.53	4.57
DY523809	ZPT2-13	5e-42	2.07	10.23	14.22	1.52	14.14	24.27
DY523446	Putative WRKY transcription factor	1e-58	2.13	4.00	7.41	1.53	3.83	16.78
DY523629	DNA-binding protein NtWRKY3	1e-107	2.45	-1.89	2.08	3.79	-1.99	-1.06
DY523772	WRKY DNA-binding protein 4	5e-57	-2.11	11.94	16.64	-1.07	9.12	9.28
DY523811	Double WRKY type transfactor	0.0	2.74	3.81	10.00	1.92	3.68	6.76
DY523358	Transcription factor	1e-146	4.43	5.03	3.09	2.31	5.63	3.45
DY523369	Heat stress transcription factor	1e-168	-1.84	1.63	3.66	-3.72	1.84	3.37
DY523646	DNA binding/transcription factor/ transcriptional activator	2e-40	-2.25	3.58	1.38	-2.35	2.43	
DY523673	Protein binding/transcription regulator	1e-114	-20.78	-13.40	-1.38	-35.89	-8.97	-1.58
DY523737	Transcription factor	3e-65	-2.32	-2.71	-3.61	-3.43	-3.18	-2.63
DY523854	NF-Y, CCAAT-binding HAT22; transcription factor	2e-70	-1.25	-2.94	-1.83	2.29	-1.39	-1.04

The third is the zinc finger family. Four different zinc finger proteins (DY523398, DY523450, DY523481, and DY523809) were identified to be differentially expressed at the early stage of salt treatment. DY523398 and DY523481 were constitutively induced by salt stress in both cultivars. DY523450 was up-regulated after 2 h of salt stress in LA2711, was down-regulated in the first 30 min, and then up-regulated at 2 h and 6 h in ZS-5. DY523809 was increasingly induced in both genotypes although it did not reach to 2-fold in the early stage in ZS-5.

In addition, several members of the WRKY family of transcription factor and other types of transcription factors were also identified in our array analysis.

Cell wall genes are highly modulated by salt stress

Several genes involved in cell wall metabolism were identified to be highly regulated by severe salt stress (Table 3). They are a xyloglucan endotransglucosylase-hydrolase (XTH; DY523676), a glucan endo-1,3- β -glucosidase (DY523636), and five arabinogalactan proteins (AGPs; DY523658, DY523732, DY523764, DY523818, and DY523893). All these cell wall genes were highly repressed by severe salt stress in both genotypes except for the XTH, which was not changed in LA2711 while significantly repressed in ZS-5, and one of the AGPs (DY523893), which was significantly induced by 2 h and 6 h severe salt stress and not changed at 30 min in

LA2711 while in ZS-5, its expression was significantly reduced at 30 min and not changed at 2 h and 6 h.

Genes involved in several pathways are changed in response to salt stress

Several genes encoding key enzymes in the nitrogen reduction and fixation pathway were significantly affected by severe salt stress (Table 4; Fig. 5). These genes include DY523598 (nitrate reductase), DY523535 (nitrite reductase), DY523624 (nitrite reductase), DY523333 (phenylalanine ammonia-lyase, PAL), DY523361 (PAL), DY523519 (glutamine synthetase, GS), DY523551 (GS2), and DY523370 (asparagine synthetase). PAL (DY523361) and asparagine synthetase (DY523370) were significantly up-regulated throughout the treatment in both cultivars. Another PAL (DY523333) was severely inhibited in ZS-5 at 30 min and 6 h, whereas it was not changed to a statistically significant level in LA2711 from the onset to 6 h of salt treatment. Except for those three genes, the other genes were continuously repressed in the first 6 h of salt stress.

Three genes involved in the methionine biosynthesis pathway were significantly down-regulated by severe salt stress at certain time points in both LA2711 and ZS-5 (Table 4). It was evident that these genes were repressed slower in LA2711 than in ZS-5, as they were all down-regulated by 30 min severe salt stress in ZS-5, while none of them were significantly affected by 30 min severe salt stress in LA2711.

Table 3. List of severe salt stress-responsive genes involved in cell wall metabolismSignificant difference (FDR <0.01 and fold change ≥ 2) in relative level is shown in bold.

Accession no.	Annotation	e-value	LA2711			ZS-5		
			0.5 h	2 h	6 h	0.5 h	2 h	6 h
DY523676	Xyloglucan endotransglucosylase-hydrolase XTH9	1e-171	-1.20	-1.54	1.02	-11.00	-3.88	-2.60
DY523636	Glucan endo-1,3- β -glucosidase B precursor	0.0	-1.69	-12.37	-3.42	-3.31	-15.42	-4.10
DY523893	Arabinogalactan protein precursor	4e-48	-1.30	2.15	3.00	-3.33	1.76	1.33
DY523732	Arabinogalactan protein	7e-60	-1.47	-17.35	-5.86	-3.19	-33.33	-21.98
DY523764	Fasciclin-like AGP 12	3e-79	-1.97	-2.94	-2.25	-2.14	-2.71	-2.65
DY523658	Fasciclin-like arabinogalactan protein (FLA10)	1e-146	-1.10	-4.16	-2.42	-2.52	-7.33	-36.41
DY523818	Putative fasciclin-like arabinogalactan protein FLA2	1e-131	1.04	-1.25	-3.23	-2.24	-1.14	-5.13

Table 4. List of severe salt stress-responsive genes involved in two metabolic pathways according to the KEGG pathway database (KEGG: Kyoto Encyclopedia of Genes and Genomes :<http://www.genome.jp/kegg/pathway.html>)Significant difference (FDR <0.01 and fold change ≥ 2) in relative level is shown in bold.

Accession no.	Putative function	e-value	LA2711			ZS-5		
			0.5 h	2 h	6 h	0.5 h	2 h	6 h
Nitrogen metabolism: reduction and fixation								
DY523519	Glutamine synthetase	0.0	-3.15	-7.71	-18.35	-2.16	-21.22	-9.33
DY523551	Glutamine synthetase GS2	1e-44	-3.43	-4.60	-20.89	-1.99	-16.98	-16.38
DY523598	Nitrate reductase	0.0	-9.90	-3.74	-5.24	-11.67	-7.20	-8.08
DY523535	Nitrite reductase	0.0	-5.57	-6.41	-8.29	-8.35	-5.84	-9.87
DY523624	Nitrite reductase	5e-22	-8.14	-16.29	-59.11	-10.46	-27.29	-27.93
DY523333	Phenylalanine ammonia-lyase (PAL)	1e-170	-1.30	-1.91	1.20	-2.99	-1.01	-2.04
DY523370	Asparagine synthetase	8e-93	4.11	3.48	5.54	2.81	4.53	6.14
DY523361	Phenylalanine ammonia-lyase	2e-83	3.26	6.52	6.02	2.84	7.33	7.72
Methionine metabolism								
DY523503	S-adenosyl-L-methionine synthetase	0.0	-1.47	-2.78	-5.93	-4.92	-1.65	-9.00
DY523617	S-adenosylmethionine synthase	0.0	-1.37	-1.58	-2.24	-2.99	-1.25	-3.74
DY523544	Methionine synthase	0.0	-1.49	-1.70	-3.92	-2.37	-2.04	-6.78

Discussion

In this work, SSH and microarray approaches were used to enrich and identify salt tolerance-related genes from both salt-tolerant and -sensitive tomato cultivars. This study focused on the early stages of severe salt stress in root tissues due to the important roles of early response genes in mediating the effects of salt stress. A total of 201 unique genes significantly changed (fold change ≥ 2 and FDR <0.01) upon 30 min severe salt stress either in LA2711 or ZS-5 were isolated. The differential expression of subset genes upon salt stress was further confirmed by RT-PCR analysis.

Phenotypic performance and screening of tomato salt tolerance

LA2711 was confirmed as a salt-tolerant tomato genotype at both germination and vegetative stages. Under salt stress, LA2711 has a final germination percentage that was significantly higher than that of ZS-5, which is a salt-

sensitive genotype. In addition, LA2711 germinated much faster than ZS-5. Distinct genetic variations were reported in different germplasms of tomato with respect to germination under salt stress, and rapid germination under salt stress could be used as a criterion for salt tolerance (Foolad and Lin, 1999). It is presumably true that for LA2711, rapid germination may contribute to salt tolerance to some extent. It was reported previously that both stress-specific and stress-non-specific quantitative trait loci were identified to contribute to seed germination of tomato under salt stress (Foolad *et al.*, 1999).

Due to the possible differences of salt tolerance between seed germination and vegetative stages (Zhang *et al.*, 2003), the response of LA2711 to salt stress at the seedling stage was also investigated. It was possible to conclude that LA2711 is a salt-tolerant genotype according to a series of physiological indices, especially its high K^+/Na^+ ratio in the leaf compared to ZS-5, which is probably one of the key determinants of plant salt tolerance (Maathuis and Amtmann, 1999).

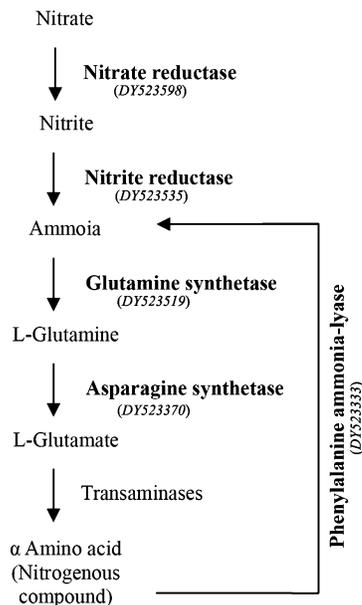


Fig. 5. Modulation of genes encoding enzymes involved in nitrogen reduction and fixation pathway by salt stress. Genes significantly modulated by salt stress are shown in bold.

SSH and microarray approaches and identification of salt-responsive genes

Salt tolerance is a complex quantitative trait regulated by a large number of genes. Exploitation and enrichment of salt tolerance-related genes is very important to accelerate the research in this area. SSH approach has been proved to be a powerful tool to enrich the differentially expressed genes (Diatchenko *et al.*, 1996). In this study, over 90% of the up-regulated genes were derived from the forward libraries, and around 80% of the down-regulated genes from the reverse libraries. However, one major problem of the SSH approach is that it usually generates quite a few false positives and so further screening of SSH generated clones is required. cDNA microarrays can monitor the expression of thousands of genes simultaneously, and thus could be an ideal choice for screening SSH clones. The approach of combining SSH and cDNA microarrays for the rapid identification of differentially expressed genes has been proved to be very efficient. This approach was adopted here to identify salt tolerance-related genes.

Transcriptional regulation and tomato salt tolerance

Twenty-four genes identified by microarray analysis encode transcription factors (Table 2). Most of these genes were first reported to be associated with salt stress in this study. Among this group of transcription factors, four belonged to the NAC protein family. The NAC protein family is one of the largest families of plant-specific transcription factors (Olsen *et al.*, 2005). Genes from this family were found to participate in various biological processes including development, biotic and

abiotic stress (Hegedus *et al.*, 2003; Guo *et al.*, 2005a). An *Arabidopsis* NAC gene, *AtNAC2*, was found to be involved in the salt stress response and in lateral root development (He *et al.*, 2005). It is worth noting that one NAC gene, DY523377, identified in this study, is exactly the one identified by Selth *et al.* (2005), which can interact with tomato leaf curl virus replication accessory protein and enhance viral replication. Thus, it is possible that this tomato NAC gene plays an important role in the cross-linking of different signalling pathways.

Six genes belonging to the EREBP family of transcription factors were also among the group of salt tolerance-related genes identified in this study. It has been reported that EREBP genes, for example, DREB1 (Liu *et al.*, 1998) and JERF1 (Zhang *et al.*, 2004), are involved in salt tolerance. Furthermore, members from the EREBP family may play important roles in the cross-talk of different kinds of abiotic stress and signalling pathways, in the respect that they are highly regulated by various kinds of abiotic factors and plant hormones, such as low temperature, ethylene, drought, high salinity, abscisic acid, and jasmonate (Liu *et al.*, 1998, 2006; Zhang *et al.*, 2004). Besides the EREBP genes identified in this study, an ACC oxidase (DY523334), a key enzyme in ethylene biosynthesis, is also highly regulated by salt stress (see Supplementary Table S2 at *JXB* online). This fact indicated that the plant hormone ethylene might play an important role in tomato salt tolerance.

Four tomato genes from the family of zinc finger transcription factors were highly induced by severe salt stress. The association between zinc finger transcription factors and plant salt tolerance has been reported previously. In particular, the expression of a rice zinc-finger protein, OSISAP1, and two *Arabidopsis* Cys2/His2-type zinc-finger proteins, AZF2 and STZ, is strongly induced by different types of stresses, including high salt treatment (Mukhopadhyay *et al.*, 2004; Sakamoto *et al.*, 2004). Overexpression of OSISAP1 in transgenic tobacco conferred tolerance to cold, dehydration, and salt stress at the seed-germination/seedling stage (Mukhopadhyay *et al.*, 2004).

The relationship between other putative salt tolerance-related transcriptional factors identified in this study, such as WRKY, HSF (Table 2), and salt tolerance, has not previously been documented. However, the discovery of various families and different members of transcription factors in this study indicated that a complicated transcriptional regulation network is involved in tomato responses upon high salt treatment.

Cell wall metabolism and tomato salt tolerance

Several genes related to cell wall metabolism were identified in this study; most of these genes were down-regulated by salt stress in both LA2711 and ZS-5 (Table 3). High salinity has significant impacts on plant cell wall metabolism by changing cell wall elasticity and

composition (Mustarda and Renaulta, 2004). It has been reported that the activities of several enzymes involved in cell wall metabolism were significantly changed by salt stress (Thiyagarajah *et al.*, 1996; Takeda and Fry, 2004).

Two cell wall genes identified in this study are of great interest. One gene, DY523676, encodes XTH and the other, DY523893, encodes AGP. Both genes responded to severe salt stress differently in the sensitive genotype than they did in the tolerant genotype. XTH cleaves and religates xyloglucan polymers, an essential constituent of the primary cell wall, and thereby participates in cell wall elongation and construction (Fonseca *et al.*, 2005). AGPs are components of cell walls and plasma membranes and are considered to play roles in cell differentiation, development, cell-cell interactions (Li and Showalter, 1996), seed germination, and seedling growth in tomato (Lu *et al.*, 2001). The different responses to salt stress in LA2711 and ZS-5 of the two tomato genes mentioned above indicated that they might contribute significantly to the salt-tolerant nature of LA2711.

Whole plant adaptation under salt stress

Genes found responsive to 30 min severe salt stress in this study belong to a total of 14 different functional groups. The functional diversity of severe salt stress-responsive genes indicated that tomato salt tolerance is a very complex trait and the whole plant engages in fighting against the stress.

A large number of differentially expressed genes identified in this study belong to the metabolism group (see Supplementary Table S2 at *JXB* online). Most genes in this group were down-regulated under severe salt stress, especially at a later stage (6 h), indicating the collapse of the plant under severe salt stress. In particular, genes involved in the metabolic pathways of nitrogen reduction and fixation and methionine biosynthesis were significantly affected by salt stress (Table 4; Fig. 5). Several key genes upstream of nitrogen reduction and fixation pathway, for example, nitrate reductase, nitrite reductase, and glutamine synthetase, were significantly down-regulated. Decreased nitrate reductase activity in total nitrogen and nitrate uptake under salt stress have been reported previously for other plants (Baki *et al.*, 2000; Parida and Das, 2004). The activity and transcript abundance of ferredoxin-dependent glutamate synthase, which is the key enzyme of nitrogen assimilation and biosynthesis of amino acids, decreased in leaves in response to salt stress in common iceplant (Popova *et al.*, 2002). These observations indicated that salt stress has significant impacts on nitrogen reduction and fixation in plants.

Three genes involved in methionine biosynthesis were identified in this study to be down-regulated by salt stress (Table 4). The association between salt tolerance and methionine biosynthesis has been discovered in yeast

(Gläser *et al.*, 1993). Increased gene dosage of HAL2, an inositol phosphatase involved in methionine biosynthesis, improves yeast growth under salt stress. In addition, methionine supplementation improves the tolerance of yeast to NaCl (Gläser *et al.*, 1993).

Despite the observation that the majority of the genes in the metabolism group were repressed by salt stress, several interesting genes such as asparagine synthetase, histidine decarboxylase, PAL, mono-oxygenase, and *S*-adenosylmethionine decarboxylase (SAMDC), were induced by salt treatment, at least at the later stages. These observations are consistent with previous reports for other plants. For instance, wheat TaASN1, an asparagine synthase, and AhCMO, a choline monooxygenase from *Atriplex hortensis*, were dramatically induced by salt stress (Shen *et al.*, 2002; Wang *et al.*, 2005). Transgenic tobacco overexpressing AhCMO conferred salt tolerance (Shen *et al.*, 2002). SAMDC is an essential enzyme for the biosynthesis of polyamines, which was reported to have specific roles in salt tolerance (Roy and Wu, 2002). Increased expression of SAMDC under salt stress suggested that proline accumulation might help tomato plants fight against salt stress. PAL and mono-oxygenase are oxidative related and can play roles in the alleviation of damage from secondary oxidative stress caused by salt stress (Lee *et al.*, 2003).

Recently in *Arabidopsis*, through the identification of *salt overly sensitive (sos)* mutants and the cloning and characterization of the *SOS* genes, a novel signalling pathway called the SOS pathway that mediates ion homeostasis and salt tolerance was discovered. In this pathway, SOS3, a calcineurin B-like calcium-binding protein, perceives the calcium signal elicited by salt stress, and subsequently transmits it to SOS2, a serine/threonine protein kinase. SOS2 and SOS3 physically interact with each other and activate SOS1, a putative plasma membrane Na⁺/H⁺ antiporter, via phosphorylation (reviewed in Zhu, 2002). In this study, two tomato calcium-binding proteins and several protein kinases were identified. Unexpectedly, no Na⁺/H⁺ antiporters were found in this study although several other kinds of transporters were significantly changed by severe salt stress. However, a cDNA encoding putative plasmalemma Na⁺/H⁺ antiporter was isolated from tomato recently (Belver and Donaire, unpublished results), although its function requires further investigation. This finding indicated that a conserved SOS signalling pathway might exist in tomato to regulate plant responses to salt stress.

Several key components, such as protein phosphatase 2C (DY523345) and MAPKKK (DY523508) in the MAP kinase signalling pathway were identified to be up-regulated at the later stages upon severe salt stress. In *Arabidopsis*, a MAP kinase signalling cascade regulating salt stress tolerance has been identified. The MAPKKKs, MAPKKs, and MAPKs identified so far in this pathway

are highly activated by salt stress (Teige *et al.*, 2004). Some protein phosphatase 2Cs (PP2Cs) are negative regulators of MAPK signalling pathways, and the increased expression of PP2Cs under salt stress could be part of a negative feedback mechanism (Meskiene *et al.*, 2003).

A gene encoding a potassium channel (DY523762) was found among the differentially expressed transcripts. A rice potassium channel, OsAKT1, was also reported to be repressed by salt stress (Fuchs *et al.*, 2005). It is crucial for plants under salt stress to maintain a low Na⁺/K⁺ ratio. In this study, the results showed that LA2711, the salt-tolerant genotype, had lower Na⁺/K⁺ ratio than ZS-5, the salt-sensitive genotype (Table 1). However, the tomato potassium channel gene identified here was dramatically down-regulated in both genotypes. This occurrence indicated that genes, possibly including other potassium channels, contributing to lower Na⁺/K⁺ ratio of LA2711 under salt stress, still remain to be identified.

It is worthy to note that among salt-modulated genes, several heat shock proteins (HSPs) were identified. HSPs, which act as molecular chaperons, play a crucial role in protecting plants against stress by re-establishing normal protein conformations, and thus, maintain cellular homeostasis (Wang *et al.*, 2004). Transgenic tobacco overexpressing DnaK1, a member of Hsp70 from the halotolerant *Cyanobacterium aphanotheca* showed increased tolerance to salt stress (Sugino *et al.*, 1999).

A large number of early response genes regulated by salt stress in this study encode unknown proteins, indicating that there is still a great deal to discover with regard to the mechanism of the salt tolerance in tomato. With the initialization and progress of the tomato whole genome sequencing and functional genomics projects (Mueller *et al.*, 2005; Fei *et al.*, 2006), more and more information regarding the tomato genome and its expression will be collected. This progress will bring to the research community a huge wealth of information on novel salt tolerance-related genes.

Supplementary data

Supplementary data in the form of two tables and one figure can be found at *JXB* online. Table S1: Primer sets list of genes for the RT-PCR analysis. Table S2: A complete list of differentially expressed genes response to salt stress in tomato. Fig. S1: Characterization of 5-week seedlings of a salt-tolerant genotype LA2711 and sensitive genotype, ZS-5, under high salt stress.

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