Comparative Transcriptomic Profiling of a Salt-Tolerant Wild Tomato Species and a Salt-Sensitive Tomato Cultivar

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Wild halophytic tomato has long been considered as an ideal gene donor for improving salt tolerance in tomato cultivars. Extensive research has been focused on physiological and quantitative trait locus (QTL) characterization of wild tomato species in comparison with cultivated tomato. However, the global gene expression modification of wild tomato in response to salt stress is not well known. A wild tomato genotype, Solanum pimpinellifolium ‘PI365967’ is significantly more salt tolerant than the cultivar, Solanum lycopersicum ‘Moneymaker’, as evidenced by its higher survival rate and lower growth inhibition at the vegetative stage. The Affymetrix Tomato Genome Array containing 9,200 probe sets was used to compare the transcriptome of PI365967 and Moneymaker. After treatment with 200 mM NaCl for 5 h, PI365967 showed relatively fewer responsive genes compared with Moneymaker. The salt overly sensitive (SOS) pathway was found to be more active in PI365967 than in Moneymaker, coinciding with relatively less accumulation of Na+ in shoots of PI365967. A gene encoding salicylic acid-binding protein 2 (SABP2) was induced by salinity only in PI365967, suggesting a possible role for salicylic acid signaling in the salt response of PI365967. The fact that two genes encoding lactoylglutathione lyase were salt inducible only in PI365967, together with much higher basal expression of several glutathione S-transferase genes, suggested a more effective detoxification system in PI365967. The specific down-regulation in PI365967 of a putative high-affinity nitrate transporter, known as a repressor of lateral root initiation, may explain the better root growth of this genotype during salt stress.

Keywords: Ion homeostasis • SABP2 • Salt tolerance • Transcriptomic profiling • Wild tomato.

Abbreviations: CAT, catalase; CBL, calcineurin B-like protein; CIPK, CBL-interacting protein kinase; FDR, false discovery rate; MeSA, methyl salicylate; QTL, quantitative trait locus; ROS, reactive oxygen species; SA, salicylic acid; SABP2, salicylic acid-binding protein 2; SOD, superoxide dismutase; SOS, salt overly sensitive.

Introduction

High salinity is a prevalent abiotic stress limiting the productivity and the geographical distribution of plants. During the past decade, our understanding of plant response to salt stress has been greatly improved by studying model plants. Salt tolerance is believed to be affected by many different genes involved in different pathways, such as ion compartmentation, ion extrusion, ion selectivity, compatible solute synthesis and reactive oxygen species (ROS) scavenging (Blumwald et al. 2000, Zhu 2001, Zhu 2003, Munns and Tester 2008). Although many of the mechanisms are probably universal in most plants, their relative importance in salt tolerance may vary from species to species, depending on the metabolic background. To find the most suitable mechanisms for improving salt tolerance of a particular crop, wild species closely related to that crop with relatively high salt tolerance might be ideal gene donors.

Cultivated tomato, one of the most important vegetable crops in the world, is moderately sensitive to salinity. The existence of halotolerant accessions of several wild tomato species has made tomato a model crop for comparative studies on the mechanisms of salt tolerance. Previous investigations have focused on the physiological and genetic characterization of wild tomato species in comparison with tomato cultivars.

Several mechanisms were proposed to contribute to salt tolerance of wild tomato species, such as ion extrusion and distribution, antioxidative mechanisms and osmoregulation. Tal and Shannon (1983) found that Solanum cheesmanii behaves like a halophyte with the ability to accumulate Na+ in the upper part of the plant. However, in another report, the salt tolerance of S. cheesmanii was suggested to be due to its ability to exclude Na+ from expanded leaves. This discrepancy may be the result of different seed batches and a different
set-up of the experiments (Rajasekaran et al. 2000). Cuartero et al. (1992) reported that the high relative growth of Solanum pimpinellifolium 'PE-2' in saline conditions was correlated with its high increase in succulence and high Na⁺/K⁺ ratio. The salt tolerance of Solanum lycopersicum accession PI174263 relative to UCTS was attributed to its ability to maintain a high tissue Ca²⁺ level and to exclude Na⁺ from the shoot (Foolad 1997).

An effective antioxidative system has also been found to be responsible for the salt tolerance of some wild tomato species. For example, the increase of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities, and the increased level of the reduced form of ascorbate and glutathione correlated with the decreased lipid peroxidation in a salt-tolerant wild genotype, Solanum pennellii, in comparison with cultivated tomato (Shalata et al. 2001, Mittova et al. 2004). In addition, osmoregulation such as the accumulation of trigonelline, but not proline, in the root, expanding leaf and meristematic tissues was found to be correlated with the salt tolerance of S. cheesmanii (Rajasekaran et al. 2000). The concurrence of Na⁺ accumulation and the increase of succulence in salt-tolerant S. pimpinellifolium 'PE-2' also suggested the possible role of Na⁺ as an osmoticum to attract water influx (Cuartero et al. 1992).

Efforts in identifying genes responsible for salt tolerance have also been made for both wild and cultivated tomato. Using a BC1 population of a cross between a tomato cultivar and a salt-tolerant wild tomato accession, Foolad et al. (2001) identified five quantitative trait loci (QTLs) for salt tolerance during vegetative growth, located on chromosomes 1, 3, 5, 6 and 11, respectively. In a tomato cultivar Moneymaker, two loci tss1 and tss2, were found to be responsible for salt tolerance (Borsani et al. 2001a). However, genes corresponding to these loci were not identified. Recently, based on the combination of suppression subtractive hybridization (SSH) and microarray, salt-induced changes in the transcriptome profile were compared in roots of two tomato cultivars with distinct salt tolerance (Ouyang et al. 2007). However, the salt response at the transcription level of more salt-tolerant wild tomato has not been reported.

In this report, a wild genotype S. pimpinellifolium 'PI365967' and the S. lycopersicum cultivar 'Moneymaker' were compared in terms of their growth and physiological responses to salt stress. Microarray analysis using an Affymetrix tomato genome array was then performed to assess their global gene regulation by salt stress, which will serve as the first step towards understanding the mechanism underlying salt tolerance of wild tomato species.

**Results**

**Performance of PI365967 and Moneymaker under NaCl stress**

To characterize the differences in salt tolerance between PI365967 and Moneymaker, six-leaf-stage plantlets of both genotypes were treated hydroponically with different concentrations of NaCl (0, 100, 200 and 300 mM). No leaf burn, wilting or plant death occurred at the NaCl concentrations below 100 mM. When a higher concentration of salt (200 or 300 mM) was applied, plants of both genotypes were severely wilted within 30 min. However, PI365967 recovered sooner than Moneymaker (data not shown). Under 200 mM NaCl, chlorosis was obvious only in Moneymaker, although slower growth was observed in both genotypes (Supplementary Fig. S1 available at PCP online). The survival rate of Moneymaker decreased after 35 d, but none of the PI365967 plants were dying (Fig. 1).

Under 300 mM NaCl, the survival rate of PI365967 after 21 d was still higher than that of Moneymaker. Shoot and root elongation were also measured after treatment with or without 200 mM NaCl for 21 d (Table 1). Shoot growth was inhibited in both genotypes, but more severely in Moneymaker. The root elongation in Moneymaker was significantly higher than that in PI365967 under control conditions, but under salinity it was faster in PI365967 than in Moneymaker. These results indicated that PI365967 is more salt tolerant than Moneymaker.

**Physiological responses to salt stress in PI365967 and Moneymaker**

Ion balance is in most cases a major determinant of salt tolerance. The concentrations of Na⁺ and K⁺ were, therefore, measured in both shoots and roots of the plants treated with 200 and 0 mM NaCl (Table 2). Both genotypes exhibited a similar increase in Na⁺ concentration in roots under salinity stress, but Moneymaker showed a higher increase in Na⁺ concentration in shoots. This resulted in a higher increase in the [Na⁺]shoot/[Na⁺]root ratio in Moneymaker than in PI365967, suggesting that there is a mechanism in PI365967 preventing Na⁺ from being transported to, or more rapidly excluding Na⁺ from, the shoot. This may help to protect the shoot apical meristem from the damage caused by excessive salt. The K⁺/Na⁺ ratios in both leaves and roots decreased under the NaCl treatment compared with control, with a lower decrease observed in Moneymaker roots and PI365967 shoots.

In addition, accumulation of compatible organic solutes following exposure to salinity stress was also measured. Fig. 2
shows that there was a similar increase in the level of proline and soluble sugar in leaves of both genotypes after treatment with 200 mM NaCl. However, the maximum accumulation of proline and soluble sugar was much lower in PI365967 than in Moneymaker. These findings suggested that Moneymaker responded more significantly to salinity.

**Microarray expression profiling**

In order to gain a better understanding of the mechanism underlying the salt tolerance of PI365967, the transcriptome changes of PI365967 and Moneymaker after 5 h salt stress were investigated using the GeneChip® Tomato Genome Array (Affymetrix) composed of >9,200 genes. A 5 h treatment with 200 mM NaCl was used because we were particularly interested in the early responses to salinity shock.

Correlation coefficients (r values) from different replicate hybridizations were calculated as measures of biological reproducibility. They were all >0.98 (data not shown), indicating that our microarray data were reproducible, reliable and suitable for further analysis.

In general, very similar expression patterns were obtained in the salt-tolerant wild genotype, PI365967, and the moderately sensitive cultivated genotype, Moneymaker. Upon 5 h salt stress, 1,386 and 948 genes showed differential expression in Moneymaker and PI365967, respectively [false discovery rate (FDR) <0.05 and fold change ≥2] (Fig. 3). Of the up-regulated genes, 86 were only identified in PI365967, 362 were only identified in Moneymaker and 409 were identified in both genotypes (Fig. 3A). Of the down-regulated genes, 82 were only identified in PI365967, 244 were only identified in Moneymaker and 371 were identified in both genotypes (Fig. 3B). This indicated that the expression of a relatively larger number of genes changed in Moneymaker.

### Table 1 The shoot and root elongation of PI365967 and Moneymaker

<table>
<thead>
<tr>
<th></th>
<th>Shoot length (cm)</th>
<th>Relative shoot elongation</th>
<th>Root length (cm)</th>
<th>Relative root elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 d</td>
<td>21 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shoot elongation</td>
<td>Root elongation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moneymaker Na</td>
<td>12.55 ± 0.91</td>
<td>14.61 ± 0.39</td>
<td>11.82%</td>
<td>18.88 ± 1.32</td>
</tr>
<tr>
<td>Moneymaker CK</td>
<td>12.60 ± 0.86</td>
<td>30.03 ± 1.68</td>
<td></td>
<td>17.43 ± 1.20</td>
</tr>
<tr>
<td>PI365967 Na</td>
<td>13.23 ± 0.88</td>
<td>17.96 ± 2.88</td>
<td>20.68%</td>
<td>22.27 ± 1.54</td>
</tr>
<tr>
<td>PI365967 CK</td>
<td>11.85 ± 1.69</td>
<td>34.72 ± 5.79</td>
<td></td>
<td>20.81 ± 1.42</td>
</tr>
</tbody>
</table>

The seedlings were treated with 1/4 strength Hoagland solution containing 0 mM (CK) and 200 mM NaCl (Na) for 21 d. Three replicates were used in each treatment, with 15 plants per replicate. Values are expressed as the mean ± SD of three repeats. Relative elongation = [length Na (21 d) – length Na (0 d)]/[length CK (21 d) – length CK (0 d)].

### Table 2 Accumulation of Na⁺ and K⁺ in shoots and roots of PI365967 and Moneymaker treated with 200 mM NaCl (Na) or control (CK) for 7 d

<table>
<thead>
<tr>
<th></th>
<th>Shoot (mg g⁻¹ DW⁻¹)</th>
<th>K⁺</th>
<th>K⁺/Na⁺</th>
<th>Root (mg g⁻¹ DW⁻¹)</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>K⁺/Na⁺</th>
<th>[Na⁺]shoot/[Na⁺]root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺</td>
<td>K⁺</td>
<td>K⁺/Na⁺</td>
<td></td>
<td>Na⁺</td>
<td>K⁺</td>
<td>K⁺/Na⁺</td>
<td></td>
</tr>
<tr>
<td>Moneymaker Na</td>
<td>0.81 ± 0.06 (2.5×)</td>
<td>13.10 ± 1.07</td>
<td>16.25</td>
<td>0.73 ± 0.03 (1.4×)</td>
<td>9.41 ± 2.96</td>
<td>12.88</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>Moneymaker CK</td>
<td>0.32 ± 0.02</td>
<td>12.70 ± 0.41</td>
<td>39.37</td>
<td>0.51 ± 0.02</td>
<td>10.51 ± 0.89</td>
<td>20.55</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>PI365967 Na</td>
<td>0.70 ± 0.04 (1.4×)</td>
<td>10.65 ± 2.69</td>
<td>15.31</td>
<td>0.77 ± 0.07 (1.5×)</td>
<td>5.02 ± 0.66</td>
<td>6.56</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>PI365967 CK</td>
<td>0.51 ± 0.02</td>
<td>13.07 ± 0.80</td>
<td>25.88</td>
<td>0.50 ± 0.02</td>
<td>11.34 ± 0.46</td>
<td>22.61</td>
<td>1.02</td>
<td></td>
</tr>
</tbody>
</table>

Three replicates were used in each treatment, with five seedlings per replicate. Values are expressed as the mean ± SD of three independent repeats.
that the SOS pathway might be more active in PI365967 than in Moneymaker, was induced by 2.52-fold. This suggested that the proton gradient to provide energy for various transporters on plasma membrane ATPase 1, functioning in generating the membrane potential, may be important for salt tolerance in PI365967. A total of 86 genes were specifically up-regulated in PI365967. A total of 86 genes were specifically up-regulated in PI365967 versus Moneymaker at a level of ≥2-fold and an FDR <0.05.

**Verification of microarray data**

Quantitative real-time PCR was performed on seven differentially expressed genes to verify the microarray data. These genes were selected randomly from the following three groups: (i) genes up-regulated only in PI365967; (ii) genes highly expressed in PI365967 under normal growth conditions; and (iii) genes up-regulated in both genotypes. Consistent with the microarray hybridization data, the result of real-time PCR showed a similar expression pattern for the tested genes in both PI365967 and Moneymaker, indicating that there was a good agreement between the microarray data and the real-time PCR results (Fig. 4).

**Genes up-regulated only in PI365967**

Since PI365967 is more salt tolerant than Moneymaker, more attention has been paid to the genes specifically up-regulated in PI365967. A total of 86 genes were specifically up-regulated in PI365967 (Supplementary Table S2). According to the functions of gene products, these genes can be classified into several groups, including signal transduction, transcription regulation, translation regulation, glycosyl transfer, defense against stress, and metabolism. Some of the genes discussed here are listed in Table 3.

In the signal transduction group, two genes encoding salicylic acid-binding protein 2 (SABP2) were found to be up-regulated by 7.1- and 2.5 fold, respectively. The 7.1-fold up-regulation represented the highest among these 86 genes. SABP2 was first identified in tobacco with esterase activity, which functions in converting methyl salicylate (MeSA) into salicylic acid (SA) and plays important roles in systemic acquired resistance (Forouhar et al. 2005, Park et al. 2007). Three genes encoding CBL (calcineurin B-like protein)-interacting protein kinase (CIPK) also showed up-regulation by 2.13- to 4.33-fold. CBL and CIPK have been implicated in regulating cellular calcium and potassium homeostasis (Hall et al. 2000, Guo et al. 2001, Li et al. 2006, Xu et al. 2006). Another gene encoding plasma membrane ATPase 1, functioning in generating the proton gradient to provide energy for various transporters on the plasma membrane, was induced by 2.52-fold. This suggested that the SOS pathway might be more active in PI365967 than in Moneymaker.

Only two transcription factor genes were shown to be specifically up-regulated in PI365967. They encode TINY-like protein and AP2/ERF domain protein, respectively, both belonging to the DREB superfamily.

In the defense category, some peroxidase genes and lactoylglutathione lyase/glyoxalase I genes involved in antioxidation and detoxification were also identified.

**Genes highly expressed in PI365967 under normal growth conditions**

It is possible that some genes important for salt tolerance of PI365967 may not be significantly induced by salt treatment. Therefore, we also focused on the genes with higher basal expression in PI365967. As shown in Supplementary Table S3, a total of 141 genes showed significantly higher basal expression (≥2-fold) in PI365967 than in Moneymaker. According to their response to salt treatment, these genes can be further divided into different groups (Supplementary Table S4).

The largest group included genes not responsive to salt stress in either tomato genotype. They were constitutively highly expressed in PI365967 and may not be induced by salt treatment. Therefore, we also focused on the genes with higher basal expression in PI365967. As shown in Supplementary Table S3, a total of 141 genes showed significantly higher basal expression (≥2-fold) in PI365967 than in Moneymaker. According to their response to salt treatment, these genes can be further divided into different groups (Supplementary Table S4).

The largest group included genes not responsive to salt stress in either tomato genotype. They were constitutively highly expressed in PI365967 although not all of them were related to the salt response. Genes encoding the Nicotiana tabacum geranylglycerolylated protein 4 (NtGP4) homolog and vacuolar H⁺-ATPase A2 subunit belonged to this group. They may contribute to the salt tolerance of PI365967.

The second largest group consisted of genes up-regulated only in Moneymaker. These genes might need to be induced
in Moneymaker under salt treatment to reach a similar expression level in PI365967. Among them, there were several genes encoding glutathione S-transferase and small heat shock protein. These genes may have lost their high expression capacity in Moneymaker during domestication due to altered environmental conditions, but can be induced in response to salinity.

Nineteen genes were induced in both tomato genotypes by salinity, including some typical salt-responsive genes, such as wax synthase, carotene β-hydroxylase, glutamate decarboxylase, iron SOD and late embryogenesis-abundant (LEA) protein genes.

A relatively small number of genes were differentially expressed in PI365967, while remaining unchanged in Moneymaker upon salt treatment, in agreement with our notion that Moneymaker was more responsive to salinity than PI365967.

### Genes up-regulated in both PI365967 and Moneymaker

There are a relatively large number of genes up-regulated in both Moneymaker and PI365967 (data not shown). Comparison of the fold induction of these genes in both genotypes revealed that the majority of the genes (346 out of 409) were induced at similar magnitude, suggesting that these two tomato genotypes shared considerable common responses to salt stress. Fifty-six genes were induced more in PI365967 than in PI365967 by salt treatment, while only seven genes were induced more in PI365967 (Supplementary Table S5). These seven genes encode putative vicilin, water channel protein, cytochrome P450-dependent monoxygenase, putative trypanothione-dependent peroxidase and cell wall metabolism-related products such as glucan endo-1,3-β-d-glucosidase and glycine-rich protein.

### Genes which were down-regulated or had lower basal expression

Eighty-two genes were down-regulated by salt treatment only in PI365967, including those related to photosynthesis, Chl metabolism, general metabolism, cell division and growth (Supplementary Table S6). This may reflect the general inhibitory effect of salt stress. The most repressed (8-fold) gene in PI365967 (unchanged in Moneymaker) among these 82 genes encodes putative high-affinity nitrate transporter, which was previously reported as a repressor of lateral root initiation (Little et al. 2005). Down-regulation of this gene during salt stress may be associated with better root growth in PI365967.

A total of 118 genes showed significantly lower basal expression in PI365967 than in Moneymaker under normal growth conditions, among which a gene encoding DELLA protein GAI was expressed at a level 7-fold lower in PI365967 than in Moneymaker (Supplementary Table S7). Upon salt treatment, it was regulated similarly in both genotypes. DELLA protein was reported as a suppressor for gibberellic acid-mediated plant growth and also plays a role in integrating environmental signals to plant growth (Achard et al. 2006). Lower GAI expression in PI365967 may be a reason why this genotype shows less growth retardation under salt stress.

A relatively large number of genes were down-regulated in both Moneymaker and PI365967 (data not shown).

### Table 3: List of selected genes up-regulated (fold change ≥2) only in PI365967 under salt stress (FDR test, q-value<5%)

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Annotation</th>
<th>PI fold</th>
<th>q-value (%)</th>
<th>MM fold</th>
<th>q-value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LesAffx.57437.1.S1_at</td>
<td>Salicylic acid-binding protein 2 [Nicotiana tabacum].</td>
<td>7.095</td>
<td>0</td>
<td>1.866</td>
<td>0</td>
</tr>
<tr>
<td>LesAffx.66270.1.S1_at</td>
<td>CBL-interacting protein kinase 21 [Populus trichocarpa].</td>
<td>4.334</td>
<td>0</td>
<td>1.586</td>
<td>0.020</td>
</tr>
<tr>
<td>LesAffx.57437.2.S1_at</td>
<td>Salicylic acid-binding protein 2 [Nicotiana tabacum].</td>
<td>2.494</td>
<td>0</td>
<td>1.693</td>
<td>0</td>
</tr>
<tr>
<td>LesAffx.67629.1.S1_at</td>
<td>CBL-interacting protein kinase 22 [Populus trichocarpa].</td>
<td>2.465</td>
<td>0</td>
<td>1.755</td>
<td>0</td>
</tr>
<tr>
<td>Les.2576.1.A1_at</td>
<td>CBL-interacting protein kinase 22 [Populus trichocarpa].</td>
<td>2.134</td>
<td>0</td>
<td>1.586</td>
<td>0</td>
</tr>
<tr>
<td>Les.1281.1.A1_at</td>
<td>Putative receptor-like serine/threonine kinase [Arabidopsis thaliana].</td>
<td>2.095</td>
<td>0</td>
<td>1.562</td>
<td>0</td>
</tr>
<tr>
<td>LesAffx.12586.1.A1_at</td>
<td>TINY-like protein 1 [Populus trichocarpa].</td>
<td>3.079</td>
<td>0</td>
<td>1.562</td>
<td>0</td>
</tr>
<tr>
<td>LesAffx.20450.1.S1_at</td>
<td>AP2/ERF-domain protein [Solanum tuberosum].</td>
<td>2.642</td>
<td>0</td>
<td>1.584</td>
<td>0</td>
</tr>
<tr>
<td>Les.1665.1.S1_at</td>
<td>Lactoylglutathione lyase family protein/glyoxalase I family protein [Arabidopsis thaliana].</td>
<td>3.378</td>
<td>0</td>
<td>1.762</td>
<td>0.005</td>
</tr>
<tr>
<td>LesAffx.57363.1.S1_at</td>
<td>Cell wall peroxidase [Capsicum annuum].</td>
<td>2.678</td>
<td>0.109</td>
<td>1.362</td>
<td>0.394</td>
</tr>
<tr>
<td>Les.3671.1.S1_at</td>
<td>Plasma membrane ATPase 1 (proton pump 1) [Solanum lycopersicum].</td>
<td>2.516</td>
<td>0</td>
<td>1.075</td>
<td>0.656</td>
</tr>
<tr>
<td>LesAffx.3918.1.S1_at</td>
<td>Cytosolic ascorbate peroxidase [Vitis vinifera].</td>
<td>2.228</td>
<td>0</td>
<td>1.752</td>
<td>0.005</td>
</tr>
<tr>
<td>LesAffx.51774.1.S1_at</td>
<td>Lactoylglutathione lyase family protein/glyoxalase I family protein [Arabidopsis thaliana].</td>
<td>2.015</td>
<td>0</td>
<td>1.692</td>
<td>0</td>
</tr>
</tbody>
</table>
Comparison of the fold inhibition of these genes by salt treatment in both genotypes revealed that the majority of the genes (349 out of 371) were inhibited at a similar magnitude, again suggesting a considerable overlap in salt responses in these two tomato genotypes. Among the commonly suppressed genes in both genotypes, the expression of 21 genes was more strongly inhibited in Moneymaker than in PI365967 by salt treatment (Supplementary Table S8), including some genes involved in cell wall formation or degradation, and peroxidase genes. This coincided with the severe wilting phenotype of Moneymaker upon salt stress. The only gene more inhibited in PI365967 (Supplementary Table S8) encodes putative protein TRANSPARENT TESTA 12 which may be involved in vacuolar transport of flavonoids (Debeaujon et al. 2001, Marinova et al. 2007).

Discussion

Phenotypic and transcriptomic characterization of PI365967 and Moneymaker

Wild tomato has long been considered as an ideal donor of genes for improving salt tolerance of cultivated tomato. To make better use of this resource, it is necessary to understand the mechanisms underlying its salt tolerance. In this report, the salt response of a salt-tolerant wild tomato, *S. pimpinellifolium* 'PI365967', was characterized, at the phenotypic, physiological and transcriptomic level, in comparison with a cultivated tomato Moneymaker. PI365967 was selected from >20 tomato genotypes based on their performance under salt stress at both germination and vegetative stages (data not shown). These genotypes included some salt-tolerant accessions which have been reported previously, such as LA0716 (Darvasi and Soller 1992, Saranga et al. 1992, Foolad and Jones 1993) and LA2771 (Ouyang et al. 2007). In our conditions, PI365967 outperformed, especially at the seedling growth stage, all the above-mentioned salt-tolerant accessions. PI365967 has been recorded as a salt-tolerant accession in the Tomato Genetics Resource Center (TGRC, http://tgrc.ucdavis.edu), and its salt tolerance was briefly described in a report of the Tomato Genetic Cooperative (http://tgc.ifas.ufl.edu/vol40/v40p14.html). However, no detailed experimental data were available.

Generally speaking, tomato is most sensitive to salt stress at the seedling growth stage, and stress tolerance at this stage is important for agriculture practice. Therefore, all our experiments were carried out at this stage. When 200 mM NaCl was applied, PI365967 showed a fast recovery from initial wilting probably resulting from osmotic shock, suggesting that PI365967 may also be more tolerant to osmotic or drought stress. During salt treatment, PI365967 could generate more new roots (data not shown), which may be one of the important attributes for its better performance.

In order to compare the salt response of PI365967 and Moneymaker at the transcriptome level, microarray analysis was performed using treatment of six-leaf-stage seedlings with 200 mM NaCl. In response to salt stress, the differential regulation of a relatively larger number of genes was observed in Moneymaker compared with PI365967. This, together with more dramatic accumulation of proline and soluble sugars, suggested that Moneymaker is more responsive to salt stress signals.

A previous study on salt cress proposed that the salt-tolerant plant had a smaller number of salt-regulated genes because of the constitutive and active overexpression of stress-related genes even under unstressed conditions (Taji et al. 2004). However, another microarray analysis indicated that a salt-sensitive wild tomato accession *S. cheesmanii* ‘LA0317’, treated in the same way, still showed a relatively smaller amount of salt-responsive genes than Moneymaker which is more salt tolerant than LA0317 (data not shown). These findings suggested that the degree of salt tolerance may not be directly linked to the extent of transcriptome modification by salt stress.

SA signaling

SA is well known as a critical hormone signal involved in the activation of both local and systemic resistance responses (Dempsey et al. 1999, Dong 2001, Glazebrook 2001, Kunkel and Brooks 2002). In such a process, SABP2 plays an important role (Kumar et al. 2006, Park et al. 2006). SABP2 was first characterized in tobacco where it was shown to bind specifically to SA with high affinity. Structural and functional analysis predicted that SABP2 is a lipase belonging to the fold hydrolase superfamily (Kumar and Klessig 2003). Later, it was demonstrated that SABP2 may be required for converting MeSA to SA (Forouhar et al. 2005). MeSA was also proposed as a mobile signal for systemic acquired resistance (Park et al. 2007).

The SABP2 gene was up-regulated only in PI365967 by salt treatment (Table 3), suggesting a potentially important role for SA signaling in the salinity response of this wild tomato genotype. The involvement of SA in abiotic stress is not well known, although foliar SA application was reported to increase the growth and relative water content, and reduce electrolyte leakage of cucumber plants grown under salt stress (Yildirim et al. 2008). SA can also improve the growth and photosynthesis of sunflower during salt stress (Noreen and Ashraf 2008). However, contradictory results have also been reported whereby SA was involved in the plant salt response by mediating the generation of ROS during salt and osmotic stresses, causing oxidative damage to Arabidopsis seedlings (Borsani et al. 2001b). Measurement of SA and MeSA content upon salt stress in PI365967 is worth carrying out to verify whether SA signaling can mediate the abiotic stress response, as it does the biotic stress response.

Ion homeostasis and the SOS pathway

Maintaining ion homeostasis is critical for the survival of plants under salinity stress. Several genes involved in sodium transport have been proven to be important for plant salt tolerance, such as SOS1, SOS2, SOS3 and AtNHX1 (Halter et al. 2000, 2002).
ROS scavenging and detoxification

Many different environmental stresses including salinity can generate ROS that are detrimental to cells at high concentrations because they cause oxidative damage to membrane lipids, proteins and nucleic acids (Smirnoff 1993, Gomez et al. 1999, Hernandez et al. 2001). Plants employ antioxidants (e.g. ascorbate, glutathione and carotenoids) and detoxifying enzymes, such as SOD, CAT and enzymes in the ascorbate–glutathione cycle (Zhu 2002), to combat oxidative stress. In our experiments, several peroxidase genes were specifically up-regulated in PI365967, although some other peroxidase genes were also up-regulated in Moneymaker. During stress, the concentration of some toxic by-products, such as methylglyoxal, also tends to increase (Yadav et al. 2005). Interestingly, two lactoylglutathione lyase genes involved in detoxification of methylglyoxal (Korithoski et al. 2007) were induced only in PI365967. Additionally, the basal expression of several glutathione S-transferase genes was found to be substantially higher in PI365967, while they needed to be induced to reach an equivalent level in Moneymaker. Therefore, it is very likely that PI365967 has a more active detoxification system.

Other identified genes

It has been reported that unlike most plant species where proline accumulation mainly resulted from enhanced biosynthesis from glutamate, dramatic elevation of free proline content in tomato during salt stress was not correlated with the expression of either of its P5CS genes (Fujita et al. 1998). In our results, although proline was significantly accumulated, the induction of the genes encoding bifunctional Δ1-pyrroline-5-carboxylate synthetase (P5CS) and γ-glutamyl kinase by salt stimulus was not observed in either tomato genotype (data not shown). Several probe sets for proline dehydrogenase were also included in the chip, and one of them was found to be down-regulated by a similar fold in both tomato genotypes (Supplementary Table S7), suggesting that reduced degradation might partially contribute to proline accumulation. Interestingly, a probe set for ornithine cyclodeaminase, which was reported to catalyse the conversion of ornithine to proline...
(Trovato et al. 2001), was found to be up-regulated similarly by 2.5-fold in both genotypes. Therefore, it is possible that the altered proline biosynthetic pathway is activated during stress.

Down-regulated genes were found to be mostly related to growth, development and general metabolism, reflecting the detrimental effects of salt stress on plants. However, some negative regulators of plant development, such as genes encoding putative high-affinity nitrate transporter and DELL protein, were found to be expressed less in salt-tolerant PI365967 after salt treatment. Their effects on salt tolerance need to be investigated further.

Moreover, there were almost 50% of total regulated genes on the chip encoding unknown proteins, some of which may encode putative proteins with particular roles in salt tolerance. Our result clearly indicated that salt tolerance of wild tomato can be the manifestation of several physiological processes.

Materials and Methods

Plant materials and growth conditions

Moneymaker, a moderately sensitive cultivar, and PI365967 (obtained from Tomato Genetics Resource Center, Davis, CA, USA), a salt-tolerant wild genotype, were used for the studies.

Seeds of uniform size were surface-sterilized by soaking in 70% (v/v) ethanol for 2 min, then in 2.63% (w/v) NaOCl for 30 min, and finally rinsed five times with sterile distilled water. The seeds were sown in a Petri dish containing 10 ml of double-distilled H₂O and germinated under 16 h light (1,500 lux) for 30 min, and finally rinsed five times with sterile distilled water. The seeds were sown in a Petri dish containing 10 ml of double-distilled H₂O and germinated under 16 h light (1,500 lux) for 30 min, and finally rinsed five times with sterile distilled water. The seeds were sown in a Petri dish containing 10 ml of double-distilled H₂O and germinated under 16 h light (1,500 lux) for 30 min, and finally rinsed five times with sterile distilled water.

For determination of ion content, six-leaf-stage plants were transferred to aerated hydroponic tanks containing 1/4 strength Hoagland solution for salt treatment, 4-week-old plants were transferred to aerated hydroponic tanks containing 1/4 strength Hoagland solution in a greenhouse. After acclimatizing for 3 d, tomato plants having six true leaves were treated with fresh 1/4 strength Hoagland solution supplemented with 0, 100, 200 and 300 mM NaCl. The survival rate was recorded for control and NaCl-treated plants in parallel after 0, 21 and 35 d. Root length was measured after 21 d. Three replicates were performed for each treatment with 15 tomato plants per replicate.

Physiological analysis

For determination of ion content, six-leaf-stage plants were treated with 200 mM NaCl for 7 d, their roots and shoots were sampled separately and dried for 2 d in an oven at 80 °C. From 0.5 to 1 g of dry samples were digested in nitric acid, and the Na⁺ and K⁺ concentrations were determined by flame photometry essentially as described (Asch et al. 2000). Three replicates were used, with five plants per replicate.

Free proline and soluble sugar content were measured in the fourth leaf from the shoot apex of plants treated with 200 mM NaCl for 0, 1, 3 and 5 d, according to Bates et al. (1973) and Dubois et al. (1956). Three replicates were used, with three plants per replicate.

RNA extraction and quantification

Total RNA was isolated from whole plants using a hot phenol method (Kay et al. 1987), examined by gel electrophoresis and quantified with UV spectrometry.

Microarray hybridization and data analysis

Microarray hybridization was performed at the CapitalBio Corporation, Beijing, PR China. Nine six-leaf-stage tomato plants were divided into three biological replicates and treated hydroponically under sterile conditions with 200 mM NaCl (salt) or 0 mM NaCl (control) for 5 h. RNA isolated from three individual pooled plants was used in hybridization to one chip, resulting in 12 chips in total (three replicates of PI365967 salt, PI365967 control, Moneymaker salt and Moneymaker control, respectively). A 10 μg aliquot of total RNA was first reverse transcribed using a T7 oligo(dT) promoter primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA was purified and served as a template in the subsequent in vitro transcription reaction. The in vitro transcription reaction was carried out in the presence of T7 RNA polymerase and a biotinylated ribonuclease mix for cRNA amplification and biotin labeling. The biotinylated cRNA targets were then cleaned up, fragmented and hybridized to GeneChip® Tomato Genome Arrays (Affymetrix). Each hybridization was performed in three duplicates. Immediately following hybridization, the probe array underwent an automated washing and staining protocol on the fluidics station 450. Then arrays were scanned with an Affymetrix® GeneChip® Scanner 3000, and the resulting images were analyzed with Affymetrix® GeneChip® Operating Software (GCOS 1.4).

A dChip normalization method was employed to normalize the ratio values. Normalized ratio data were then log transformed. Differentially expressed genes were identified using SAM software, and multiple test corrections were performed using the FDR (Benjamini and Hochberg 1995). Genes with an FDR ≤0.05 and a fold change (i.e. ratio value) ≥2 were identified as differentially expressed genes. The annotation of probe sets was obtained from either Affymetrix or the Molecule Annotation System (MAS, http://bioinfo.capitalbio.com/mas/) of CapitalBio Corporation. The microarray data and the related analysis information from this work were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE16401.

Quantitative real-time PCR analysis

Total RNA isolated from 5 h of both stressed and control plants were used for real-time PCR analysis. First-strand cDNA was synthesized from 5 μg of total RNA with oligo(dT) and Superscript™ reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. The reverse transcription
mix was diluted five times and 1 µl was used for quantitative real-time PCR, carried out with gene-specific primers, using an MX3000P instrument and a SYBR® Green Realtime PCR Master Mix (QPK-201; Toyobo). Quantification was performed using the $2^{-\Delta\Delta Ct}$ method and the data were normalized for the quantity of actin transcript. All the specific primer sequences are shown in Supplementary Table S1.

### Supplementary data
Supplementary data are available at PCP online.

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### References


