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Abstract. **Tomato (*Solanum lycopersicum* cv. Money Maker) seedlings at the two-leaf stage were grown in one-half strength Hoagland solution supplemented with 50 mM NaCl for 4 days, with 100 mM NaCl for 4 days, with 150 mM NaCl for 4 days, and with a final concentration 200 mM NaCl for 2 days. Solutions were refreshed every 2 days for treated and untreated seedlings. Non-treated plants were grown in nonamended one-half strength Hoagland solution. Three biological replicates (BR) were included for treated and control experiments. At the end of treatments, the uppermost three newly expanded leaves from all 12 plants in each BR were collected and bulked to extract total protein. Proteomic analysis resulted in the identification of several salt-induced and salt-suppressed proteins. Salt-induced proteins were: vacuolar H^+-ATPase A1 subunit isoform (1.6-fold), germin-like protein (1.5-fold), ferredoxin-NADP (+) reductase (1.2-fold), quinone oxidoreductase-like protein (4.4-fold), heat-shock protein (4.9-fold), and pyrophosphorylase (1.7-fold). Salt-suppressed proteins were: ATPase alpha subunit (–1.5-fold) and rubisco activase (–1.4-fold). Proteins identified in this study affect cellular activities for antioxidant, stress protection, carbon fixation, and carbohydrate partitioning in young tomato leaves under salt stress.**

Soil salinity is a worldwide threat to irrigated vegetable and other crop production. Under saline conditions, the monovalent Na^+ cation competes mainly with K^+ due to their similar valance structure, and interferes in normal cellular processes (Fonseca et al., 2007). In addition to being toxic, NaCl can cause osmotic stress and deficiency of essential nutrient elements (Zhu 2003).

The cultivated tomato is classified as a moderately salt-sensitive plant (Katerji et al., 2003). When grown in saline media, tomato production is reduced by 50% when the saturation extract reaches an electrical conductivity (EC) of 7.5 $dS \cdot m^{-1}$ (Caro et al., 1991). Physiological studies with different tomato cultivars grown in high salt solutions indicate that Na^+ ions are transported and accumulated in leaf tissues, and consequently become inhibitory to photosynthetic activities in sensitive genotypes (Dehan and Tal, 1977).

The ability of plants to maintain low cytosolic sodium concentrations is controlled by their ability to selectively absorb Na^+ and K^+ by their roots, transport these ions to aboveground tissues, and exclude or compartmentalize Na^+ in foliar vacuoles. These activities are modulated through the functions of Na^+/K^+-ATPase of several transmembrane proteins (transporters and antiporters) and H^+ pumps (Fonseca et al., 2007; Zhu, 2003). Zhang and Blumwald (2001) found that overexpression of a vacuolar Na^+/H^+ antiport gene in transgenic tomato enhanced the secretion of Na^+ into vacuoles, reducing toxic cation concentrations in cytosolic spaces, which allowed these plants to grow, flower, and produce fruits when subjected to 200 mM sodium chloride. The yeast *HAL1* gene facilitates K^+/Na^+ selectivity and salt tolerance in cells. Under salt stress (100 mM), homozygous transgenic tomato lines overexpressing the *HAL1* gene retained more K^+ than wild types, but it did not improve their salt tolerance in terms of increase in fruit yields (Gisbert et al., 2000; Muñoz-Mayor et al., 2008).

The performance of tomato plants under salt stress is regulated by a complex genetic mechanism (Foolad, 2004). Plants perceive and respond to stressful conditions by quickly altering their gene expression in parallel with physiological and biochemical modulation. To adapt to salt stress, new proteins in tomato seedlings are induced (Amini et al., 2007). To maintain inner cellular osmotic status, tolerant genotypes can accumulate a higher content of inositol and sugars in their leaves (Sacher and Staples, 1985). mRNA profiling of NaCl-treated tomato plants has shown that salt stress can affect many different pathways (Ouyang et al., 2007; Zhou et al., 2007).

The objective of this study was to identify salt-regulated proteins using a comparative analysis between NaCl treated and non-treated leaves. Based on the putative functions of identified proteins, a molecular mechanism for salt tolerance/sensitivity is proposed.

Material and Methods

Plant growth conditions and salt treatments. 'Money Maker' tomato seeds (Rachel's Supply, Gautier, MS) were surface disinfected by submergence in 2.62% containing sodium hypochlorite (Dolgencorp, Goodlettsville, TN) for 10 min followed by three washes in sterile double-distilled H_2O. Germinated seeds were planted in sterile perlite and placed in

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an incubator at 25 °C until one leaf on each seedling emerged (20 d). Six uniform-sized seedlings were then transferred to each of the 12 Styrofoam containers (20 × 20 × 15 cm) for treated and nontreated replicates. Three biological replicates, each consisting of 12 plants growing in two Styrofoam containers, were included for treated and control experiments. These containers were filled with one-half strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) and placed on greenhouse benches under natural light at 25/21 °C (day/night). The nutrient solutions were aerated continuously during the experiment and the levels were adjusted daily and replaced with fresh solution every 2 d. A completely randomized design was used.

Salt treatments were initiated when seedlings in each biological replicate (BR) had two fully expanded leaves. They consisted of maintaining test plants in a hydroponic solution supplemented with increasing NaCl (Fisher Scientific, Pittsburgh) concentrations for 4 d with 50 mM, 4 d with 100 mM, 4 d with 150 mM, and for 2 d with a final concentration of 200 mM. Nontreated (control) plants were grown in one-half strength Hoagland's nutrient solutions. At the end of the treatments, the uppermost three fully expanded leaves were harvested from the 12 plants in each BR, bulked, and frozen in liquid N_2. For protein extraction, frozen leaves were ground to a fine powder under liquid N_2 and suspended in an extraction buffer (1:3, v/w) that consisted of 75 mM Tris, pH 7.5, 100 mM DTT, 0.3% SDS, and 0.1% protease inhibitor cocktail (Sigma, St. Louis), and were incubated overnight at 4 °C on a rotary mixer.

Preparation of protein samples, protein quantification, and two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) analysis. To remove cell debris, protein extracts were centrifuged at 15,500 g_n at 4 °C for 10 min. Proteins in the supernatant were precipitated overnight in a 20% trichloroacetic acid (TCA) solution at 20 °C and were pelleted at 15,500 g_n. Pellets were washed five times in prechilled acetone to remove residual TCA and were air dried at 4 °C. After reconstitution in a 2D protein rehydration buffer [7 M urea, 2 M thiourea, and 4% CHAPS (Sigma) in 25 mM Tris, pH 8.6], the concentration of total protein in each sample was quantified using the Bradford protein assay (Bio-Rad, Hercules, CA). Bovine serum albumin ranging in concentrations from 0 to 1 mg·mL^{-1} was used as the standard (van Noorden et al., 2007).

To allow for detectable differences in CyDye (GE HealthCare, Piscataway, NJ) intensities, each biological repeat was labeled with Cy3 and Cy5. A dye swap was included in the experimental design to provide a technical replicate and to normalize the differences in dye reactivity. To facilitate gel-to-gel normalization, a Cy2-labeled internal standard containing equal amounts of protein from all samples was included in each gel. Dye swaps and all possible combinations of pairwise comparisons between samples were included. For the composition of samples in each gel, see Table 1.

Samples used for 2D-DIGE were initially fractionated using a Multiphor II system (GE HealthCare). For this first-dimension separation, immobilized pH gradient IPG strips (GE HealthCare) 24 cm in length with nonlinear pH 3.0 to 11.0 gradients were used. A total of six gels were run, and each one was loaded with labeled protein from nontreated plants (50 µg), treated plants (50 µg), and internal standard (50 µg) samples (150 µg in total), which were mixed with the IPG strip rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 10 mM DTT, all from Sigma) and 2% IPG strip buffer (GE HealthCare). Four hundred fifty microliters of this solution was added to each lane of each rehydration tray and left to rehydrate at room temperature for 12 h. Isoelectric focusing (IEF) was carried out at 20 °C and the voltage was increased from 300 to 3500 V during a 1.5-h period. The final voltage was set at 3500 V for 4.5 h. At the end of the IEF run, proteins were reduced and alkylated (Zhang et al., 2003).

Table 1. Composition of protein samples for two-dimensional fluorescence difference gel electrophoresis.

Gel no.	Cy3[z]	Cy5[y]	Cy2[x]
1	Salt-treatment replicate 1	Control replicate 2	STD[w]
2	Salt-treatment replicate 2	Control replicate 3	STD
3	Salt-treatment replicate 3	Control replicate1	STD
4	Control replicate 1	Salt-treatment replicate 2	STD
5	Control replicate 2	Salt-treatment replicate 3	STD
6	Control replicate 3	Salt-treatment replicate 1	STD

[z]Protein labeled with Cy3 dye.
[y]Protein labeled with Cy5 dye.
[x]Protein labeled with Cy2 dye.
[w]Internal standard containing a mixture of equal amount proteins from all salt-treated and control replicates was used in comparison for normalizing between gels.

Strips were transferred and apposed to 24 × 21-cm, 12% precast SDS-polyacrylamide gels (Jule Biotechnologies, New Haven, CT) and were run on an electrophoresis apparatus (DALT Six, GE Healthcare). The following protocol was used: 16 °C at 20 mA for 30 min, then 50 mA for 5 to 6 h until the bromophenol blue (BPB) front marker reached the bottom of the gel. For expression analysis, proteins were labeled with Cy dyes as previously described. Digital gel images were obtained using a laser scanner (Typhoon 9400; GE Healthcare) and raw image files were analyzed using the Progenesis Samespots (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Observed protein pIs were calculated using the chart of Immobiline DryStrip pH 3–10 NL 24 cM pH as function of distance at 20 °C and 8 M urea (GE Healthcare). The molecular mass was calculated based on the protein migration distance (Rf) calibrated against SDS-PAGE Molecular Weight Standards Broad Range (catalogue no. 161–0317; Bio-Rad). Theoretical mass and pIs were predicted using the tool of PeptideMass (SIB Switzerland, 2007). The protein subjected for query was the tomato unigene when the complete gene sequence was available. When the unigene contained a partial sequence, proteins with the highest identity from other species, such as arabidopsis (*Arabidopsis thaliana*), tobacco (*Nicotiana tobaccum)*, or potato (*Solanum tuberosum*), were used.

Protein spots that showed a change of at least 1.2-fold (mean of the six gel replicates) between control and treated samples with $P < 0.5$ were selected for digestion and identification by mass spectrometry (MS) analysis. For protein identification, separate picking gels were run. Picking gels were stained with colloidal Coomassie blue (NOVEX CCB staining kit; Invitrogen, Carlsbad, CA) and the protein spots were picked manually.

In-gel digestion with trypsin. In-gel digestions and tryptic peptide extractions were performed following the protocol of Shevchenko et al. (1996) with slight modifications. Gel pieces were washed and destained using a series of washes with 50 µL of water, 50 µL of 50% acetonitrile/50% (Fisher Scientific), 50 mM ammonium bicarbonate (Sigma), pH 7.8, and 50 µL of

100% acetonitrile. Once samples were completely dried, 0.2 µg of modified trypsin (Promega, Madison, WI) in 20 µL of 50 mM ammonium bicarbonate (pH 7.8)/10% acetonitrile were added to each tube. Samples were iced for 15 min and incubated overnight at 37 °C.

The recovered supernatant and remaining peptides were sequentially extracted from the gel using a series of solutions. The first one contained 50 µL of 50% acetonitrile with 2% formic acid (FA) and the second contained 50 µL of 90% acetonitrile with 0.5% FA. For each extraction, samples were sonicated for 10 min before removing the supernatant. Supernatants were combined and dried in a Speedvac (Thermo Savant, Holbrook, NY).

Protein identification by MALDI-TOF-TOF MS. Each sample was reconstituted in 3 µL of 50% acetonitrile (ACN) (Fisher Scientific) with 0.1% trifluoroacetic acid (TFA) before MS analysis and 1 µL was spotted on a MALDI target plate and immediately spotted on top with 0.5 µL of saturated matrix [10 mg·mL^{-1} α-CHCA (α-cyano-4-hydroxy cinnamic acid (Sigma)] and recrystallized in 50% ACN with 0.1% TFA and 1 mM ammonium phosphate) and was allowed to completely dry. Samples were then subjected to MALDI-TOF/TOF tandem mass spectrometry (MS/MS) analysis using a 4700 Proteomics Analyzer equipped with TOF-TOF ion optics (Applied Biosystems, Framingham, MA) with 4700 Explorer version 3.5. This instrument was operated in 1 kV reflector positive ion mode and calibrated with a calibration kit (Applied Biosystems) containing a mixture of six standard peptides as a default calibration for spectra acquisition. The laser power was set to 4600 for MS and 5200 for MS/MS with CID off. MS spectra were acquired across the mass range of 800 to 4000 Da with a minimum S/N filter at 25 for precursor ion selection. MS/MS spectra were acquired for the 20 most abundant precursor ions with a total accumulation of 2000 laser shots.

The combined MS and MS/MS data were submitted to Mascot 2.2 (Matrix Science, Boston) using GPS Explorer 3.65 for a search against the SGN-annotated database (SOL Genomics Network, 2007). The search parameters allowed for one missed tryptic cleavage, and variable modifications of methionine oxidation and cysteine carboxyamidomethylation. When possible, the known autodigestion products of trypsin were used as internal mass calibrants. Only proteins with a >95% confidence interval (CI) are listed as identified.

Results and Discussion

Identification of salt-regulated proteins in tomato leaf tissues. Proteome analysis was used to compare leaves of NaCl-treated and nontreated tomato plants. Leaves from all BR were collected and analyzed using DIGE (Table 1). Well-resolved protein spots were selected to compare the amount of protein on 2D gels (pI range of 3–11) (Fig. 1A). Seventeen protein spots that significantly changed (analysis of variance $P \leq 0.05$, Table 2) in response to the salt treatment are shown in Fig. 1B. The change in folds for all significant ones was between –1.4 and 4.9 (Table 2).

Using MS/MS and database search, 10 spots with proteins of known identities were identified from salt-treated leaf proteome (Table 2). Identical proteins were confirmed in the corresponding protein spots (protein spot at the same position in treated and untreated protein 2D gels) of the untreated samples. Several protein spots were not identified because there were no matching proteins in the SGN-annotated database (SOL Genomics Network, 2007).

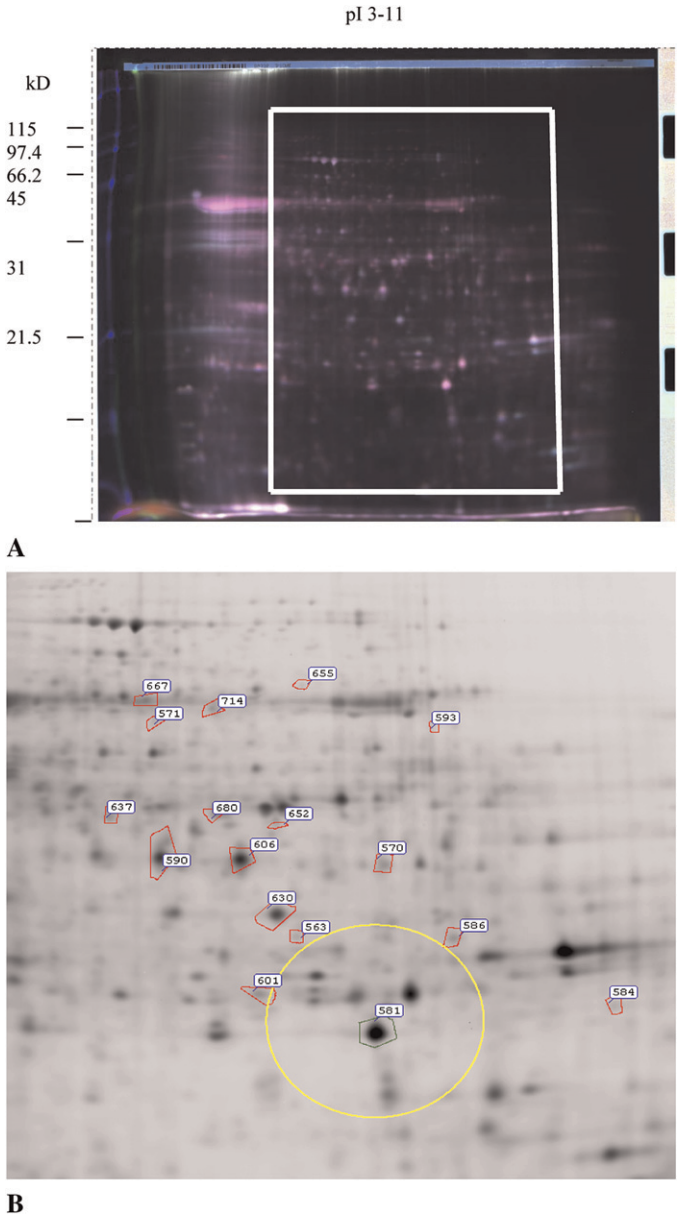

Fig. 1. Images for two-dimensional fluorescence difference gel electrophoresis (**A**) and spot picking gel (**B**) of tomato leaf protein. Gel (**A**) shows differential expression of two samples and an internal standard, labeled with Cy3, Cy5, and Cy2, respectively. Only those well-resolved spots within the height-lighted square were analyzed for protein abundance.

When compared for the theoretical and observed values of molecular mass and pI of identified proteins, spots 571 and 581 contained the proteins of which both parameters were very similar. However, for other spots, discrepancies between predicted and observed values were observed with the pI, the molecular mass, or both. The proteome is a dynamic system comprising similar proteins with alteration in surface charge or molecular weight that result from post-translational modifications, alternative splicing, etc. (Corthals et al., 2000), and these proteins migrate to different positions on a 2D gel (Marcotte 2001). This could be one of the main reasons that caused the discrepancy between theoretical and observed values.

Table 2. Identification of the salt-regulated proteins in tomato leaves.

Spot identification no.	Gene annotation	SGN identification no.[z]	Theoretical mass (kDa) [pI][y]	Observed mass (kDa) [pI][x]	Folds (salt/control)	ANOVA (*P* value)	Transcript fold[w]
563	ATPase alpha subunit	SGN-U329173	55.0 [5.1] (tomato YP514837)	37.6 [5.2]	–1.5	0.000367	NA
570	n/i[v]			35.4 [6.0]	1.2	0.000122	
571	Vacuolar H+-ATPase A1 subunit isoform	SGN-U315094	70.0 [5.2] (tomato SGN-U315094)	70.5 [5.2]	1.6	0.000823	NA
581	24K germin like protein	SGN-U313123	22.0 [6.2] (tomato SGN-U313123)	24.2 [6.2]	1.5	0.000618	1–1-8.3.19.1 (1.43-fold)
584	n/i			28.0 [8.0]	–1.6	0.000766	
586	n/i			35.4 [6.2]	–1.4	0.00175	
590	Ferredoxin–NADP(+) reductase	SGN-U314955	40.0 [8.4] (tobacco CAA74359)	47.3 [5.4]	1.2	0.002384	1–1-6.1.9.8 (1.87-fold)
593	n/i			70.5 [6.2]	1.5	0.00206	
601	Unnamed protein product	SGN-U318899	n/i	30.0 [5.6]	1.4	5.40E-05	NA
606	Ferredoxin-NADP(+) reductase	SGN-U314955	40.5 [8.4] (tomato SGN-U314955)	51.4 [5.4]	1.2	0.000751	1–1-6.1.9.8 (1.87-fold)
630	Rubisco activase	SGN-U312544	50.8 [8.8] (tomato SGN-U312544)	45.4 [5.6]	–1.4	0.003262	1–1-2.2.2.8 (-2.5)
637	Quinone oxidoreductase-like protein	SGN-U314190	32.7 [5.9] (arabidopsis AAM62737)	52.1 [5.4]	4.4	0.020247	1–1-3.2.14.6 (2.1-fold)
652	Heat shock protein	SGN-U312609	n/i	46.6 [5.6]	4.9	0.033331	NS
655	n/i			80.8 [5.8]		0.006662	
667	n/i			75.5 [5.4]		0.001845	
680	n/i			54.3 [5.6]		0.011746	
714	UDP-glucose pyrophosphorylase	SGN-U312830	52.0 [5.7] (potato AAB71613)	75.5 [5.6]	1.7	0.002227	NA

[z]SOL Genomics Network (tomato unigene) identification number (Mueller et al., 2005).

[y]Theoretical mass and pI were predicted using the tool of PeptideMass (SIB Switzerland, 2007). The protein subjected for query was the tomato EST sequences, if the full gene sequence is unavailable, or when the unigene only contains partial sequence, the gene with the closest identity but identified in other species such as arabidopsis, or tobacco, or potato, was searched in protein database and used to determine the theoretical molecular mass and pI.

[x]The observed protein pI was calculated using the chart of Immobiline DryStrip pH 3–10 NL.24 cM pH as function of distance at 20 °C and 8 M urea (GE Healthcare, Piscataway, NJ), and molecular mass was calculated based on the protein migration distance (Rf) calibrated against Bio-Rad SDS-PAGE Molecular Weight Standards Broad Range (Cat#161–0317, Bio-Rad, Hercules, CA).

[w]Zhou et al., 2007; NA = not identified in proteomics analysis, NS = no aligned sequence on Tom 1 array.

[v]No unigene identified from the array ($P < 0.05$).

The SGN unigene IDs were cross-linked to the TOM1 cDNA microarray (Boyce Thompson Institute, 2008). When four genes were compared with transcriptional regulation patterns from a microarray analysis (Zhou et al., 2007), they had the same regulatory patterns (up or down) at the protein and transcript levels. These included the induction for the germin protein, ferredoxin-NADP (+) reductase and quinone oxidoreductase-like protein, and the reduction of rubisco activase (Table 2). The expressed sequence tags (ESTs) for other proteins were not found on microarray chips; consequently, their transcriptional activities were not determined.

Induction of thioredoxins antioxidant system in tomato leaves under salt stress. Maintaining appropriate redox status in cells is important for normal activities. Salt stress induces oxidative stress in chloroplasts and activates different antioxidant mechanisms for defense against injuries (Panda and Upadhyay, 2004; van Camp et al., 1996). The thioredoxin system reduces peroxides such as H_2O_2 to harmless products. Plant chloroplastic thioredoxin system uses ferredoxin-dependent thioredoxin reductase, while cytosolic and mitochondrial thioredoxins are reduced by NADPH thioredoxin reductase (Serrato et al., 2004). In leaves of salt-treated tomato plants, the ferredoxin-thioredoxin-reductase protein (SGN-U314955) was induced (1.2-fold). This gene was also induced at the transcript level identified in cDNA microarray analysis [Tom 1 microarray ID: 1–1-7.3.4.5, 1.79-fold (Zhou et al., 2007)].

Salt stress suppression of rubisco activase. Rubisco activase was suppressed at the protein (–1.4-fold) and the transcript (–2.5-fold) levels (Table 2). This enzyme is the initial controlling point for carbon fixation because it converts rubisco from an inactive form to an active state and acts as a chaperone for promoting and maintaining catalytic activity (Portis 2003). It also participates in the signal transduction pathway (Komatsu et al., 2003). When enzyme activity between salt-sensitive and selt-tolerant species was compared, rubisco activase was found to be induced by NaCl in the halotolerant green alga *Dunaliella salina*a (Liska et al., 2004) and in the salt-tolerant *Populus euphratica* (Gu et al., 2004). The overall reduction at transcriptional and translational levels of rubisco activase could lead to lower total enzyme activity and to lower CO_2 assimilation in tomato leaves under salt stress.

Salt stress induction of quinone detoxification. NAD(P)H:quinone oxidoreductase is an enzyme that detoxifies quinones and their derivatives (Radjendirane et al., 1998; Gaikwad et al., 2001; Schuler et al., 1999). Salt stress induces lipid peroxidation in leaves of sensitive *A. thaliana* (Cavalcanti et al., 2007), producing toxic reactive aldehydes that can be scavenged by the NAD(P)H:quinone oxidoreductase homolog (Mano et al., 2002). NAD(P)H:quinone oxidoreductase has been shown to be responsible for the detoxification of heavy metals in mammalian cells (He et al., 2006; Korashy and El-Kadi, 2006). In leaves of salt-treated tomato plants, the quinone oxidoreductase-like protein (SGN-U314190) had the most significant increase (4.4-fold) and concurrently the corresponding EST transcript (1–1-3.2.14.6) also had a 2-fold increase (Zhou et al., 2007).

Induction of UDP-glucose pyrophosphorylase and partitioning of carbohydrates under salt stress. Salt stress affects carbohydrate metabolism and partitioning in various organisms (Chen et al. 2006; Fernandes et al., 2004; Khelil et al., 2007). UDP-glucose pyrophosphorylase (UGPase) catalyzes conversion of glucose-1-phosphate and UTP into uridine diphosphoglucose (UDPG), which is a key precursor for the biosynthesis of sucrose (Meng et al., 2007), cell wall polysaccharides (Ordin and Hall, 1968), and starch (Viola et al., 2001). Salt stress enhanced the accumulation of UGPase in tomato leaves by 1.7-fold (Table 1). To determine the dominant partition of UDPG, we compared the transcript level associated with those metabolic reactions (Zhou et al., 2007). Sucrose synthase catalyzes the degradation of sucrose to UDPG in the following reaction: UDP + sucrose = UDPG + fructose. According to transcript changes, salt stress induced the sucrose synthase gene (1–1-8.4.8.6). Sucrose molecules maintain the cellular osmotic equilibrium between the intra- and extracellular environment, protecting cells from osmotic damage. Juan et al. (2005) reported that salt-tolerant tomato cultivars had a sucrose concentration almost twice that of the most sensitive cultivars. In leaves of salt-treated plants, the induction of sucrose synthase could enhance the conversion of sucrose into UDPG; however, its impact on the concentration of sucrose in leaves of salt-treated plants needs to be further studied.

In this study, most of the known genes related to cell wall synthesis were induced; these were: cellulose synthase (1–1-7.4.1.7, 3.25-fold-up), which catalyzes the synthesis of cellulose, the endoxyloglucan transferase (1–1-5.2.13.2, 1.61-fold-up), and the lignin-forming anionic peroxidase precursor (1–1-2.4.20.1, 1.68-fold-up). Extensive deposition of secondary cell wall materials prevents cell expansion (Lagrimini et al., 1987). Plant growth in the transgenic hybrid poplar (*Populus alba* × *P. grandidentata*) overexpressing a UDP-glucose pyrophosphorylase (UGPase) from *Acetobacter xylinum* was retarded and accompanied by higher cellulose contents (Coleman et al., 2007). In tomato plants, the pattern of carbohydrate partitioning appears to favor cell wall synthesis. Its impact on young leaf growth needs to be further investigated.

Conclusion

In leaves of salt-treated tomato plants, ferredoxin-thioredoxin-reductase and quinone oxidoreductase were activated to alleviate oxidative stress and to detoxify toxic molecules generated by the stress. Rubisco activase was suppressed; this could lead to reduction in carbon fixation and glucose synthesis. UDP-glucose pyrophosphorylase was induced, but the UDPG products appeared to be more likely used for the synthesis of cell wall materials instead of sucrose and starch.

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