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Effect of Heat Stress on Leaf Proteome and Enzyme Activity in *Solanum chilense*

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ABSTRACT

Two-month-old *Solanum chilense* seedlings were subjected to 40°C heat treatment for 7 days. Comparative proteomics analysis determined that heat affected the abundance level of proteins in the following pathways: photorespiration (glycolate oxidase and hydroxypyruvate reductase), secondary metabolite production (flavonol synthase and NAD-dependent epimerase/dehydratase), photosynthesis-light reaction (ferredoxin reductase and NADPH: protochlorophyllide oxidoreductase), methionine biosynthesis (homocysteine methylase), carbon fixation and metabolism (phosphoglycerate kinase and fructose-bisphosphate aldolase), and ATP regeneration (ATP synthase). The NADPH: protochlorophyllide oxidoreductase and one isoform of glycolate oxidase were induced, and all the other proteins were suppressed by the heat treatment. Enzyme activity assays were performed on two-month-old-seedlings and two-year-old-greenhouse plants subjected to 40-50°C heat treatments. In the two-month old seedlings, heat treatment suppressed hydroxyperuvate reductase activity, but glycolate oxidase activity maintained at similar levels. The two-year-old plants contained higher hydroxyperuvate reductase activity, but lower glycolate oxidase activity compared to the two-month-old seedlings. Peroxidase activity was much higher in the two-year-old plants than the two-month-old seedlings. Plants exposed to the extreme heat at 50°C showed the most dramatic and consistent changes in all of the three enzymes. This study has provided valuable information for the understanding of molecular mechanism for heat tolerance in *S. chilense*.

Keywords: enzyme activity, photorespiration, photosynthesis, proteomics, wild tomato

INTRODUCTION

Heat stress is defined as a rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to plant growth and development (Wahid *et al*. 2007). Any temperature above the optimum growth temperature will exert a stress effect on susceptible species; but the degree of injury depends on the level of stress as well as plant tolerance. Severe heat stress especially when combined with drought causes permanent impairment of the photosynthetic capacity, leading to stunted (or no) growth and low yield (Berry and Björkman 1980). Agriculture is highly sensitive to weather extremes. In 2011, the persistent extremely high heat (temperatures of 38-45°C for over 20 consecutive days) and exceptional drought (over 70 consecutive days with no rain in June-October) have caused more than $5 billion worth of damage to Texas agriculture, and additional billions in impact on crops and livestock in the southern plains and Mississippi Valley in the United States of America (USA) and Mexico (Forsyth 2011; Smith 2011). If global warming continues, more frequent and severe 'heat-waves' are expected in temperate zones, leading to hotter and drier summers thus imposing more serious threat to agricultural production (Semenov 2007; Semenov and Halford 2009; Saunders *et al*. 2011). Improving plant tolerance to heat (and drought) stress is now a major undertaking for agricultural research and development.

Tomato (*Solanum lycopersicum* Mill.) is one of the most important vegetable crops. In the USA, the total cultivated area for tomato was 178,911.5 ha producing 15,588,800 tons of fresh fruit in 2009 (The USDA Economics, Statistics and Market Information System 2010). Temperature has a significant influence on many aspects of growth and development in tomato. The optimum tomato growing temperature is 21-25°C during the day, with nights of 18-25°C (Hussey 1965; Marr 2003; van der Ploeg and Heuvelink 2005). Both the vegetative and reproductive performances of tomato are suppressed by heat stress when the ambient temperature reaches above the optimal range (Abdalla and Verkerk 1968; Dinar and Rudich 1985). High temperature induces abnormal development in pollen and female reproduction tissues and abortion of young fruits, resulting in low or no yield (Peet and Bartholemew 1996; Peet *et al*. 1997). Tomato leaves subjected to prolonged heat stress conditions experience starch depletion due to enhanced hydrolysis and inhibited biosynthetic activities (Dinar *et al*. 1983). An analysis of whole leaf proteome of tomato cultivars has shown that heat stress suppressed expression of proteins in the photosynthesis and carbon fixation pathways, which would affect plant growth due to reduced carbohydrate assimilation (Zhou *et al*. 2011).

Fig. 1 *Solanum chilense* plants growing in hot greenhouse at Nashville, Tennessee, USA.

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Tolerance to moderate heat stress has been identified in tomato varieties and wild relative species. RAPD and ISSR markers linked to heat tolerance have been found in recombinant inbred lines of tomato cultivars. These are very useful for marker assisted selection of targeted traits (Lin *et al*. 2006; Kamel *et al*. 2010). Genes encoding for heat stress proteins (Hsps), nucleocytoplasmic small heat stress proteins (Hsp17-CI, CII, and CIII), and heat shock factors class A (HsfA1) and B (HsfB1) are induced by heat stress and those proteins are involved in the molecular control of heat tolerance (Bharti *et al*. 2000; Siddique *et al.* 2003; Bharti *et al*. 2004; Port *et al*. 2004).

However, results from breeding projects have indicated that very few of the currently available tomato varieties, breeding lines, and accessions have a high level of heat tolerance (>38°C) (Villareal *et al.* 1978). The wild tomato species, *S. chilense*, has a longer primary root and more extensive secondary root system than do cultivated tomatoes (O'Connell *et al.* 2007), and it is adapted to the desert areas of northern Chile. This species is five times more tolerant of wilting than regular cultivars (de la Peña and Hughes 2007; Chetelat *et al*. 2009) and is able to survive high ambient temperature of 45-50°C (**Fig. 1**).

The objective of this study was to identify heat-responsive proteins in *S. chilense* by comparing leaf proteomes from heat treated and untreated plants. Biochemical analysis was conducted to validate the functional pathways involving the identified proteins. Results of this study will be used in genome mapping of heat tolerance traits in *S. chilense* and to identify functional and regulatory elements responsible for heat tolerance in this species.

MATERIALS AND METHODS

Plant growth and heat treatment

Seed stocks were originally obtained from C.M. Rick Tomato Genetics Resource Center, University of California, Davis, CA, USA. For this experiment, two-month-old seedlings with four fully expanded mature leaves were treated in incubators illuminated with fluorescent tubes at an irradiance level of 500 µmol m^{-2} s^{-1} with a 16-h photoperiod. For temperature treatments, the incubators were programmed at 40°C for the treated and 25°C for the control experiments. Four incubators were used, two for the control and two for the heat treatment. For proteome analysis mature leaves were collected at 13:00 pm. For enzyme analysis the same type of leaf tissues was collected daily during the 7 day treatment period. For each treatment (heat-treated or control), three biological replicates were collected, one from each of the two incubators, and the third one consisted of equal number of plants from the two incubators for the treatment. Each sample was composed of the top two mature leaves from 10 plants. The wild tomato *S. chilense* is self-incompatible, mature plants can grow continuously without forming fruits if not pollinated by a sibling plant. The two-year-old plants were maintained in a heated greenhouse. Leaves were collected for three consecutive days when the greenhouse temperature reached 40 or 50°C, respectively. Immediately after collection, tissues were frozen in liquid nitrogen and processed for protein extraction.

Preparation of protein samples and DIGE (differential two dimensional (2D) fluorescence gel electrophoresis) analysis

DIGE analysis was performed on leaf proteins from the two-month-old seedlings treated in the incubators. Protein extraction and DIGE analysis followed the procedure previously described (Zhou *et al*. 2011). Briefly, leaf tissues were ground into a fine powder under liquid nitrogen, and total protein was precipitated in acetone containing 10% trichloroacetic acid (TCA) and 0.5% dithiothreitol (DTT) (Sigma, St. Louis, MO, USA). Protein was precipitated after centrifugation at 10,000 × *g* at 4°C for 10 min following overnight incubation at -20°C, protein pellets were washed four times with pre-chilled 100% acetone. Dried protein pellets were re-swollen in 2D protein rehydration buffer consisting of 7 M urea, 2 M thiourea and 4% 3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS). Soluble proteins were separated by centrifugation at 14,000 × *g* for 10 min.

For DIGE analysis, proteins of treated and untreated samples were labeled with cyanine dyes Cy3 and Cy5 (GE Healthcare, Piscataway, NJ, USA) with dye swaps to control for labeling biases. A combined Cy2-labeled internal standard containing equal amounts of all the protein extractions used in the experiment was used to normalize across the multiple gels. The dye: protein ratio for the experiments was 200 ρmol dye: 50 µg total protein. The analytical gels were run using 50 µg of protein from each labeled sample. Proteins were separated on the first dimension by isoelectric focusing (IEF) on the 24 cm Immobiline DryStrip pH 3-10 nonlinear (NL) (GE Healthcare), and then the second dimension on 12.52% acrylamide -SDS gels (255 × 196 × 1 mm).

Gels were scanned on a Typhoon 9300 Variable Mode Imager (GE Healthcare) and gel images were analyzed using Progenesis Samespots (version 3.3, Nonlinear Dynamics, Newcastle Upon Tyne, UK). Spots (picking lists) were selected as being differentially expressed if they showed greater than a 1.5-fold change in spot density and an analysis of variance (ANOVA) score of $P < 0.05$.

For protein identification, preparative picking gels were run in which 450 µg of protein was loaded. Gel preparation and electrophoresis were done following the same procedure as DIGE gels. The protein gels were stained with Colloidal Blue Staining solution (Invitrogen, Carlsbad, CA, USA) overnight and destained in ddH_2O. Proteins spots that were matched to the DIGE spots were picked manually from the gels and digested *in situ* with trypsin (sequence-grade trypsin; 12.5 ng mg^{-1}; Promega, Madison, WI, USA) overnight. The resulting peptides were extracted from the gel pieces and concentrated with ZipTip C_{18} pipette tips (Millipore, Bedford, MA, USA). An aliquot of each digest was spotted (along with matrix) onto a MALDI-MS (matrix assisted laser desorption/ionization- mass spectrometry) target.

MALDI analysis was done on a 4700 Proteomics Analyzer equipped with TOF-TOF (time-of-flight) ion optics (Applied Biosystems, Framingham, MA, USA). The MS data were processed using Mascot Daemon (Matrix Science) to submit searches to Mascot (version 2.3, Matrix Science, Boston, MA, USA). The search parameters used were as follows: tryptic protease specificity, one missed cleavage allowed, 30 ppm precursor mass tolerance, 0.5 Da fragment ion mass tolerance with a fixed modification of cysteine carbamidomethylation and a variable modification of methionine oxidation. Spectra were searched against an in house tomato protein database (Thannhauser, unpublished data) created by combining 40,000 predicted proteins from the tomato Unigene build 2 (Sol Genomics Network, Ithaca, NY, USA) release (3/25/2009) and 9,000 predicted proteins that to date had been annotated in the tomato genome (release 5/3/2009). Only peptides that matched with a Mascot score above the 95% confidence interval threshold ($P < 0.05$) were considered for protein identification. Only proteins containing at least one unique peptide (a sequence that had not been previously assigned to different protein) were considered.

Enzyme extraction and activity assay

Except as noted, all chemicals used were obtained from Sigma (St. Louis, MO, USA). For enzyme extraction, leaf tissue samples were ground into a fine powder under liquid nitrogen. The powder (200 mg) was re-suspended in 2 ml of prechilled 50 mM potassium phosphate buffer (pH = 7.5) with 1 mM polyethyleneglycol (molecular weight: 8000 Da), 8% polyvinylpyrolydone (molecular weight: 40,000 Da), and 0.01% Triton X-100. Protein extraction was carried out by incubating the mixture for 9 h at 4°C while being constantly mixed on a rotary mixer. After centrifuging the sample twice at 12,000 × *g* and 4°C for 10 min, the clear supernatant containing the enzymes was used immediately for analysis.

Glycolate oxidase was assayed essentially as described by Feierabend and Beevers (1972) by the increase of absorbance at 324 nm every min for 20 min. The assay mixture contained 33 mM triethanoleamine buffer, pH 7.8; 2.7 mM EDTA, pH 7.8; 0.0083% Triton X-100; 0.2 mM flavin mononucleotide (FMN); 3.3 mM phenylhydrazine-HCl, pH 6.8 and 5 mM glycolic acid

(neutralized to pH, 7.0 with 1 M NaOH). Glycolate oxidase activity was determined by following the formation of glyoxylate phenylhydrazone (extinction coefficient of 17 mM^{-1} cm^{-1}).

For hydroxypyruvate reductase, the reaction mixture contained 25 mM potassium phosphate buffer, pH 5.8; 0.01% Triton X-100, 0.2 mM NADH; 1 mM dithiothreitol and 20 mM sodium glyoxylate. After the addition of NADH, the absorbance at 324 nm was measured every min for 20 min. Enzyme activity was determined by following the oxidation of NADH (extinction coefficient of 6.2 mM^{-1} cm^{-1}) (Booker *et al*. 1997).

Peroxidase assay mixture contained 0.1 M potassium phosphate buffer pH 7.0, 18 mM guaiacol, 0.136 mM hydrogen peroxide prepared from 30% hydrogen peroxide stock (9.79 M). The absorbance at 436 nm was measured every min for 20 min. Unit definition (purpurogallin unit) is the amount of enzyme which catalyzes the conversion of one micromole of hydrogen peroxide per minute (Bergmeyer 1974).

Protein concentration was determined using the Biorad Protein Assay reagent (Biorad, Hercules, CA, USA). A standard curve was constructed with bovine serum albumin (BSA). All absorbance measurements were performed on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA), using Costar 3635 UV transparent plates (Corning Inc., Corning, NY, USA). The absorbance in each well was normalized to a pathlength of 1 cm using the factory-stored values in the SOFTMAX® PRO SOFTWARE (Version 5, Molecular Devices, Sunnyvale, CA, USA).

RESULTS

Proteins induced or suppressed by heat treatment (40°C) in leaves of *S. chilense*

DIGE analysis resulted in the identification of 21 protein spots whose intensity correlated with exposure to heat stress (**Fig. 2**), 14 spots were down-regulated and 7 up-regulated. All protein spots were picked from SDS-PAGE gels and proteins contained in those spots were subjected to MALDI-TOF-TOF analysis. Eighteen protein spots were associated to known proteins after database search. Based on the putative functions of identified proteins, eight cellular processes were affected by the heat treatment (**Table 1**).

In the photorespiration pathway, three proteins were identified, which were glycolate oxidase (GOX) (spot 466, -2.1-fold; spot 459, 1.7- fold), hydroxypyruvate reductase (HPR) (spot 324, -2.4-fold), and catalase (spot 465, -1.4-fold). The two proteins in the secondary metabolite biosynthesis pathway were flavonol synthase (spot 573, -2.2-fold; spot 290, -1.9-fold), and NAD-dependent epimerase/dehydratase in the brassinosteroid biosynthesis (spot 405, -1.6-fold). In the photosynthesis pathway, two proteins were identified: the heat suppressed ferredoxin-NADP (+) reductase (FNR) (spot 404, -2.3-fold), and the heat-induced NADPH: protochlorophyllide oxidoreductase, a key enzyme in chlorophyll biosynthesis (spot 564, 2.2-fold). The homocysteine methylase in the methionine biosynthesis was suppressed by heat treatment (spot 158, -2.4-fold). Elongation factor 1-alpha (spot 116, 1.9-fold) which affects protein translation was suppressed. For carbon fixation and metabolism pathways, the phosphoglycerate kinase (PGK) (spot 541, -1.6-fold) was suppressed, and two spots were identified to be fructose-bisphosphate aldolase (spot 584, -1.6-fold; spot1050, -1.5-fold). Plants have two organelle-specific PGK and fructose-bisphosphate aldolase isoenzymes, they are localized in chloroplast participating in the Calvin cycle carbon fixation, or the cytosol participating in the glycolytic pathway (Patron *et al.* 2004). The ATP synthase protein which is a key protein in ATP regeneration was suppressed (spot 1059, -1.8-fold).

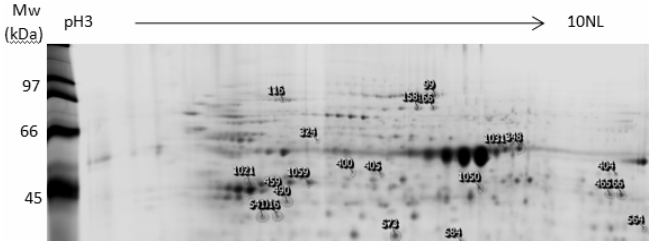


Fig. 2 The difference gel electrophoresis *(*DIGE) electropherogram of leaf proteomes in heat treated *Solanum chilense*. Two-month-old seedlings were treated for 7 day at 40°C and the control was at 25°C. Proteins were labeled with cyanine dyes using the CyDye DIGE Fluors (GE Healthcare, Piscataway, NJ, USA). Proteins from control and treated samples were labeled with Cy3 and Cy5 and an internal control consisting equal amount of all protein extractions was labeled with Cy2. The Cy-dye-labeled proteins (150 µg total with 50 µg from each of the three labeled samples) were focused on 24-cm Immobiline DryStrips pH 3-10 nonlinear (NL) [GE Healthcare (shown on the X-axis)] and then separated on 12.52% acrylamide–sodium dodecyl sulphate gels. The molecular weight markers (Mw) are shown on the Y-axis; they were the Cy2-labeled Broad Range Protein Molecular Weight Markers (Bio-Rad, Hercules, CA, USA). Gels were scanned on a Typhoon 9300 Variable Mode Imager (GE Healthcare). NL: non-linear; Mw: molecular weight.

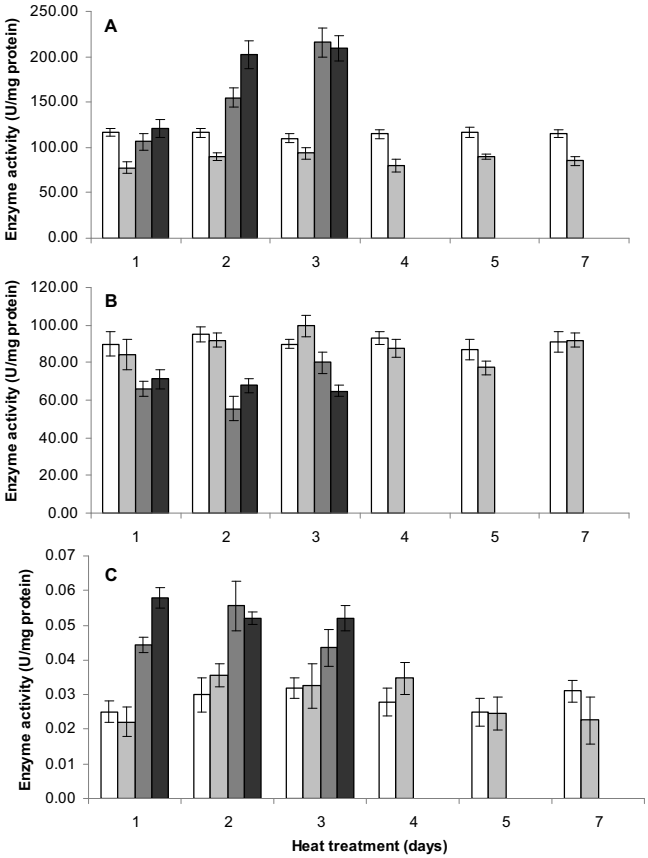
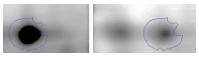
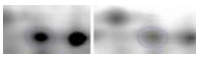
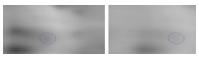
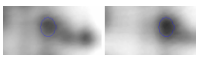


Fig. 3 Effect of heat stress on enzymatic activity in leaf tissues of *Solanum chilense.* Two-month-old seedlings were treated at 40°C and the control was at 25°C, and the top-two mature leaves were harvested daily for 7 consecutive days. The two-year-old plants were grown in a greenhouse and leaf tissues were collected when ambient temperature reached 40 ± 1°C at 9-10:00 am and 50 ± 1°C at 12-13:00 pm. Absorbance of NADH-hydroxypyruvate reductase was measured at 324 nm, glycolate oxidase at 324 nm, and peroxidase at 436 nm. The absorbance was recorded every min for 20 min, and the change in absorbance per min in the linear range was used to calculate unit of enzyme activity/mg protein. All absorbance measurements were performed on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA), using Costar 3635 UV transparent plates (Corning Incorporated, Corning, NY, USA). The absorbance in each well was normalized to a pathlength of 1 cm using the factory-stored values in the SOFTMAX® PRO SOFTWARE (Version 5, Molecular Devices). Standard error bars indicate standard deviation among the three replicates for each treatment. (**A**) NADH-hydroxypyruvate reductase; (**B**) glycolate oxidase; (**C**) peroxidase. Values represent mean ± standard error (SE). □: Incubator at 25°C; □: Incubator at 40°C; □: Greenhouse at 40°C; ■: Greenhouse at 50°C.

Effect of heat stress on enzyme activity in leaves of *S. chilense*

For the two-month-old seedlings, the NAD-HPR activity was consistently lower in the heat treated leaves than in the control during the 7 d treatment period. In the two-year-old plants exposed to 40-50°C heat, the first day measurement of enzyme activity was at a similar level to the two-month-old untreated control plants, it increased significantly on the second day, especially at 50°C, which continued during the third day under the 40-50°C treatments (**Fig. 3A**). The GOX activity showed no significant difference between the heat-

Table 1 Heat-regulated proteins in leaves of wild tomato *Solanum chilense*. Only circled dots are identified proteins.

Treatments		Spot no	UniGene ID[z]	Putative protein	Fold[y] change (Anova[x], p<0.05)	Peptide sequence hit[w]
25°C	40°C					
Photorespiration						
		466	SGN-U578941	Glycolate oxidase (GOX)	-2.1	IPVFLDGGVR; LAVQAGAAGIIVSNHGAR; FVLPPFLTLK; ALALGASGIFIGRPVVFSLAAE; GEAGVKK; QLDYVPATISALEEVVK
		459	SGN-U578941	GOX	1.7	LAVQAGAAGIIVSNHGAR; IPVFLDGGVR
		324	SGN-U578941	Hydroxypyruvate reductase	-2.4	GPVIDEVALVEHLR; FVTAYGQFLK; MNLIYYDLYQSTR
		465	SGN-U578479	Catalase 2	-1.4	EGNFDLVGNNFPVFFIR; GFFEVTHDIAHLTCADFLR; LGPNYLQLPANAPK; TWPEDILPLQPVGR; APGVQTPVIVR; FSTVIHER
Secondary metabolites						
		573	SGN-U592241	Flavonol synthase	-2.2	TSIGTFICPHEIVEPAK; SHCIPEHERPSDPVEIGDSIPVIDLGK; EVIGAYGDELRR; TWPDNPPR
		490	SGN-U592241	Flavonol synthase	-1.9	TSIGTFICPHEIVEPAK; SHCIPEHERPSDPVEIGDSIPVIDLGK; EVIGAYGDELRR
		405	SGN-U581327	NAD-dependent epimerase/dehydratase	-1.6	VVGTQAPVELGSLR
Photosynthesis						
		404	SGN-U581081	Ferredoxin-NADP(+) reductase	-2.3	DPNATVIMLATGTGIAPFR; GVCSNFLCDLKPGAEVK; DNTFIYMCGLK; KAEQWNVEVY; ITGDDAPGETWHMVFSTEGEVPYR; GMEQGIDEIMSSLAER
		564	SGN-U577510	NADPH:protochlorophyllide oxidoreductase	2.2	EPSFTAEGFELSVGTNHL; GHFLLSR
Methionine biosynthesis						
		158	SGN-U577720	Homocysteine methylase	-2.4	YGAGIGPGVYDIHSPR; YLFAGVVDGR; GVTAFGFDLVR; DEAFFSANAAAQASR
Protein translation						
		116	SGN-U578520	Elongation factor 1-alpha	-1.9	YYCTVIDAPGHR; IGGIGTVPVGR; MIPTKPMVVETFAE; YPPLGR
Carbon fixation and carbon flow						
		541	SGN-U580583	Phosphoglycerate kinase	-1.6	LASLADLYVNDAFGTAHR; FLKPSVAGFLLQK; ADLNVPLDDSQNITDDTR; YSLAPLVPR; KLASLADLYVNDAFGTAHR
		584	SGN-U577720	Fructose-bisphosphate aldolase	-1.6	SAAYYQQGAR; IVDVLLEQNIVPGIK
		1050	SGN-U578125	Fructose-bisphosphate aldolase	-1.5	SAAYYQQGAR; RLDSIGLENTEANR; ATPEQVADYTLK; GLVPLAGSNNESWCQGLDGLASR
Cellular energy (ATP) regeneration						
		1059	SGN-U577570	ATP synthase beta chain 1	-1.8	SSANAGAGSGGGPISR

[z] Unigene ID: unigene in the SOL Genomics Network (Ithaca, NY) database.
[y] Fold change value is the ratio of the normalized volume of the same spot in the condition of heat-treated (growing at 40°C) versus control (25°C). For example, a value of 2.0 represents a two-fold increase, whereas -2.0 represents a two-fold decrease from treated to control conditions.
[x] ANOVA (*P*): The *P* value in an analysis of variance test of difference between treated and untreated samples each with three biological replicates.
[w] Peptide sequence used in Mascot search to identify the protein in protein database.

treated and untreated two-month-old seedlings, but the two-year-old plants contained lower enzymatic activity, moreover, the 50°C heat treatment resulted in the lowest enzyme activity (**Fig. 3B**). The two-year-old plants growing in the hot greenhouse (40-50°C) contained much higher peroxidase activity than the two-month-old plants in the incubator experiment at both the control and heat treated conditions (**Fig. 3C**).

DISCUSSION

Candidate heat tolerance proteins in *S. chilense*

Heat stress induces damages to photosynthetic apparatus and disruption of metabolic pathways (Berry and Björkman 1980; Weis and Berry 1988). For the light reactions of photosynthesis, functional and intact PSI and PSII centers are required to smoothly convert solar energy into the energy rich molecules, ATP (in PSI) and NADPH (in PSII). FNR catalyzes the last enzymatic step of the noncyclic photosynthetic light reaction responsible for the reduction of $NADP^+$ in the PSI complex, generating the NADPH needed for CO_2 fixation and other biosynthetic routes (Hurley *et al*. 2006). Kubien and Sage (2008) showed that the electron transport pathway of photosynthesis is highly susceptible to severe temperatures above 40°C. In *S. chilense*, the FNR protein level was suppressed by the 40°C heat treatment, additionally this protein was suppressed by heat stress in heat tolerant tomato cultivar 'Walter' and drought tolerant cultivar 'Edkawi' (Zhou *et al*. 2011). These results indicate that FNR is heat-labile and independent of genetic background of cultivated tomato and the wild relative species. In contrast, the NADPH: protochlorophyllide oxidoreductase (POR) protein was induced by heat stress in *S chilense*. Protochlorophyllide oxidoreductase catalyzes the light-dependent reduction of protochlorophyllide. Research in *Arabidopsis thaliana* suggests that this enzyme contributes to photoprotection during greening of the etiolated seedlings (Masuda *et al*. 2003). The heat-induced expression of POR protein in *S. chilense* could be an important mechanism to alleviate heat-induced photodamage to the photosynthetic apparatus.

Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) activase (RCA) is a chaperone protein, modulating Rubisco activity (Spreitzer and Salvucci 2002; Portis 2003). RCA constrains the photosynthetic potential of plants at high temperatures and endogenous levels of RCA could serve as an important determinant of plant productivity under heat-stress conditions (Ristic *et al*. 2009; Yamori and von Caemmerer 2009). In *S. chilense*, the RCA protein did not have significant change after the heat treatment, but it was suppressed in cultivated tomatoes (Zhou *et al*. 2011). Such contrasting behavior between *S. chilense* and cultivated tomato suggests that RCA protein in *S. chilense* could play an important role in conferring heat tolerance.

Proteins in the photorespiratory pathway and heat tolerance in *S. chilense*

Photorespiration occurs when the ratio of O_2/CO_2 inside a leaf becomes low due to the closure of stomata under environmental conditions such as drought and heat. This carbon oxidation pathway plays a complex role in mediating this stress condition: it is an energy-consuming process, but also functions to prevent accumulation of toxic glycolate and depletion of intermediates from the Calvin cycle (Timm *et al*. 2008). From *S. chilense* two enzymes, NADH-dependent-HPR and GOX, in the photorespiration pathway were identified. The 40°C heat treatment suppressed HPR for both protein abundance and enzyme activity in the two-month-old seedlings. Plants contain multiple isoforms of HPR (Kleczkowski *et al*. 1988). The consistent correlation between protein abundance and enzymatic activity indicates that the HPR protein identified from DIGE gel directly affected the total enzyme activity, thus it could be the key isoform for heat stress response.

GOX was identified in two protein spots, one heat-suppressed and one heat-induced. The total enzyme activity showed no significant change after heat treatment of the two-month-old seedlings. Apparently this protein has multiple isoforms (two protein spots on DIGE gel), which were regulated differentially by heat stress. Differential regulation of these isoenzymes could be one of the heat-tolerance mechanism in *S. chilense*. This might suggest that the two isoforms are targeted to different locations within the cell or tissue. The total activity remains the same but the special distribution of the activity varies in a critical way shifting the activity to where it is needed in the stressed plant. Characterization of each isoform could help to understand the respective function for heat tolerance.

Tolerance to extreme heat in *S. chilense*

In this study, the two-year old *S. chilense* plants had grown through different seasons for two years. The heating system in the greenhouse was able to maintain temperature at above 20°C during cold season, but the cooling system was not functioning. Plants growing in the greenhouse had exposed to gradually increasing temperature, and acquired some heat tolerance. Those two-year-old plants were able to survive 5-7 consecutive days of above 50°C noon temperature in the months of June-early September. However, in this study, when the two-month-old plants were treated by the 50°C heat in incubators, leaves were severely dehydrated and dying within 2 hr. These results indicate that heat-acclimation in the two-year-old plants had significantly enhanced their thermo-tolerance. The development of heat-shock tolerance (45°C vs. 38°C) through the heat acclimation process, also known as acquired tolerance, has also been found in tomato cultivars (Camejo *et al*. 2007).

In this study, the GOX activity was stable at 40°C in the two-month-old seedlings, but it was greatly suppressed in the two-year-old plants that were treated at 40-50°C. On the contrary the HPR activity was suppressed in the two-month-old seedlings which were subjected to the 40°C treatment, but it was induced in the two-year-old plants treated at 40-50°C. Moreover, within the two-year-old plant group, the 50°C treatment induced a more dramatic increase in HPR (day 2) and reduction in glycolate oxidase (day 3). Such differential response between the two types of plant materials to moderate heat (40°C) or extreme heat (50°C) suggests that the amount of enzymes accumulated in plants was affected by environmental temperature as well as the developmental stages of plants.

Acquired tolerance to stress extremes is regulated by stress signal transduction pathways, transcriptional factors, and cell membrane stability (Sung *et al*. 2003). It is important to analyze those molecular components and physiological factors in *S. chilense* to understand the mechanisms regulating how plants from this species develop tolerance to the extremely high temperature (>50°C). In addition, the plants that were subjected to extreme heat stress for a period of two-years contained significantly higher peroxidase activity compared to those measured in two-month-old seedlings. This result suggests that higher amounts of reactive oxygen species were accumulated in those plants which induced the strongest peroxidase activity (Apel and Hirt 2004).

In conclusion, DIGE protein analysis and enzymatic activity assays show that photorespiration, photosynthesis, production and removal of ROS and toxic compounds were affected by heat stress in *S. chilense*. More information on the predicted and validated molecular markers, gene loci, gene functions, and many others associated with the identified proteins can be found by searching tomato database (Sol Genomics Network 2011). This study has provided valuable information for understanding molecular mechanism for heat tolerance in plants.

DISCLAIMER

Disclaimers of the U.S. Department of Agriculture: "Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture" and "USDA is an equal opportunity provider and employer."

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