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Tissue-Specific Defense and Thermo-Adaptive Mechanisms of Soybean Seedlings under Heat Stress Revealed by Proteomic Approach

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A comparative proteomic approach was employed to explore tissue-specific protein expression patterns in soybean seedlings under heat stress. The changes in the protein expression profiles of soybean seedling leaves, stems, and roots were analyzed after exposure to high temperatures. A total of 54, 35, and 61 differentially expressed proteins were identified from heat-treated leaves, stems, and roots, respectively. Differentially expressed heat shock proteins (HSPs) and proteins involved in antioxidant defense were mostly up-regulated, whereas proteins associated with photosynthesis, secondary metabolism, and amino acid and protein biosynthesis were down-regulated in response to heat stress. A group of proteins, specifically low molecular weight HSPs and HSP70, were up-regulated and expressed in a similar manner in all tissues. Proteomic analysis indicated that the responses of HSP70, CPN-60 β, and ChsHSP were tissue specific, and this observation was validated by immunoblot analysis. The heat-responsive sHSPs were not induced by other stresses such as cold and hydrogen peroxide. Taken together, these results suggest that to cope with heat stress soybean seedlings operate tissue-specific defenses and adaptive mechanisms, whereas a common defense mechanism associated with the induction of several HSPs was employed in all three tissues. In addition, tissue-specific proteins may play a crucial role in defending each type of tissues against thermal stress.

Keywords: proteome • soybean • tissue-specific • temperature • heat shock protein

Introduction

The growth, development, and yield of plants are affected by climatic variability via linear and nonlinear responses to weather variables, particularly temperature stress.[1] Global warming is a growing threat to all living organisms, including plants. High temperatures (above 35 °C) are an important environmental factor that has considerable influence on the growth and productivity of many crops, including soybean.[1] Experimental and crop-based models for major crops in tropical and subtropical regions showed direct yield losses in the range of 2.5−16% for every 1 °C increase in seasonal temperatures.[2]

Plant adaptation to thermal stress is dependent upon the activation of cascades of molecular networks associated by stress perception, signal transduction, and the expression of stress-related proteins.[3] Protein metabolism, including protein synthesis and degradation, is one of the most sensitive processes in heat stress. Understanding the mechanisms of plants adaptations to heat stress would facilitate the development of heat-tolerant cultivars for improving productivity in warm-climate regions. Recently, several efforts have been made to explore the heat-stress-induced proteome responses of monocotyledonous crop plants.[4−6] Moreover, the changes in protein level during heat stress has also been demonstrated in several noncrop, model plants such as *Poplus*,[7] Norway spruce,[8] and *Agrostis*.[9,10] Most of these studies investigated the proteome changes of aerial parts of plants which revealed that antioxidant and heat shock proteins (HSPs) were mostly up-regulated and proteins associated with photosynthesis were generally down-regulated in response to heat stress. Neilson et al.[11] emphasized the role and current status of proteomic technologies in understanding the molecular mechanisms of plant under thermal stresses. Although identification of several proteins has contributed substantially to our understanding of the molecular basis of the heat stress response, the mechanisms that operate in a tissue-specific manner and that are involved in adaptation of the whole plant to heat stress are still unclear due to the lack of comprehensive tissue-specific proteome analyses.

The protein contents of whole tissues and individual cells show considerable differences, while each cell of an organism has the same genome.[12] Recently, proteomic approaches revealed that a number of proteins were differentially expressed in various tissues and showed a considerable degree of variability during normal development[13] and under stressful conditions, including heat stress.[8−10] Whiteman et al.[14] demonstrated the presence of large numbers of sugar transporters

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in green tissues, whereas several ammonium transporters were exclusively expressed in roots. These results provide a good example of a conserved tissue-specific regulatory mechanism in plants. Moreover, a large number of unique proteins can be differentially regulated at various stages of development even within an organ.[13] These earlier proteomics analyses clearly revealed that plants adopt divergent organ- and tissue-specific regulatory mechanisms at different developmental stages and in response to stressful conditions. Thus, profiling of tissue-specific protein expression in plants in response to environmental stresses is valuable to plant biologists for better understanding of the tissue-specific stress responses and adaptive mechanisms to particular stresses.

Thus far, only a few studies have investigated the proteomic responses of crop-plants to heat stress, and those are mostly in monocotyledonous plants.[11] However, limited information is available on the heat-stress responses of major dicotyledonous crops and, specifically, their tissue-specific response at the proteome level. Soybean, the world's most widely grown seed legume, occupies more land globally than any other dicotyledonous crops and is considered more sensitive to heat stress than the other legumes.[15] In the present study, we aimed to investigate the proteomic responses of the leaves, stems, and roots of soybean seedlings to heat stress in order to identify the heat-induced, tissue-specific proteins and to demonstrate the tissue-specific defense strategy and thermo-adaptive mechanisms of soybean seedlings against heat stress.

Experimental Procedures

Plant Growth, Treatments, and Sample Collection. Soybean (*Glycine max* L. cv Enrei) seedlings were grown in a controlled growth chamber under white fluorescent light (300−350 μmol m^{-2} s^{-1}, 16 h light/8 h dark) at 25−20 °C ± 2 °C (day/night) temperatures and 75% relative humidity. Two-weeks old seedlings were transferred in a different growth chamber with same light/photoperiod conditions mentioned above and subjected to heat stress by exposed to 40 ± 2 °C and 75% relative humidity. First trifoliates, stems, and roots were harvested 6, 12, and 24 h after heat treatment.

A different set of seedlings was also subjected to cold and oxidative stress treatments. Cold treatment was given by transferring the seedlings in a cold growth chamber (10 °C), and leaf samples were collected 12 h after exposure. Oxidative stress treatment was applied by submergence of trifoliates in 10 mM of hydrogen peroxide (H_2O_2) solution for 12 h. Leaves submerged in Mili-Q were used as positive control. The concentration and exposure time point for H_2O_2 treatment was selected mainly based on the earlier report by Banzet et al.[16] After each treatment, samples were immediately frozen in liquid nitrogen and kept at −80 °C until analysis. Samples collected from three different biological replicates were used for proteomic analyses.

Protein Extraction and Polyethylene Glycol Fractionation. Total soluble proteins were extracted from leaf stem and root samples as described previously.[13] A portion (1 g) of fresh sample was ground in liquid nitrogen and homogenized in 10 mL of ice-cold Mg/NP-40 extraction buffer containing 0.5 M Tris-HCl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% (v/v) β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, and 0.7 M sucrose. An equal volume of Tris-HCl saturated phenol (pH 8.0) was then added and mixed well by vigorous vortexing for 2 min followed by centrifugation at 3500× *g* for 15 min. After centrifugation, the top phenol phase was collected and proteins were precipitated by mixing with four volumes of cold methanol containing 0.1 M ammonium acetate at −30 °C for 2 h.

In order to remove the high abundant proteins ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from the leaf samples, proteins were extracted with Mg/NP-40 extraction buffer followed by fractionation with polyethylene glycol (PEG, 4000, Wako, Osaka, Japan) as described by Kim et al.[17] with slight modification. A portion (1 g) of leaf sample was ground in liquid nitrogen and homogenized in 10 mL of ice-cold Mg/NP-40 extraction buffer except adding sucrose, followed by centrifugation at 3500× *g* for 15 min. The proteins in the supernatant were subjected to PEG fractionation by adding 15% (w/v) ice-cold PEG solution. After a gentle mix, sample was incubated on ice for 30 min and then centrifuged at 12 000× *g* for 15 min at 4 °C. The supernatant fraction was further fractionated with 15% (w/v) ice-cold PEG solution as above. After centrifugation, the supernatant was recovered and precipitated with acetone and stored in 1 mL aliquots at −30 °C until use.

2-DE and Image Acquisition. The protein pellet was air-dried and resuspended with solubilization buffer containing 8.5 M urea, 2.5 M thiourea, 5% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, 1% dithiothreitol (DTT), 1% Triton X-100, and 0.5% Biolyte (pH 5−8, Bio-Rad, Hercules, CA). The solubilized protein was quantified using an RC-DC Protein Assay kit (Bio-Rad) and bovine serum albumin as the standard. For each sample, a total of 350 μg of solubilized proteins were applied to the IPG dry strips pH 5−8 (11 cm, ReadyStrip, Bio-Rad) with 12 h rehydration following the manufacturer's instruction. IEF was performed using PROTEAN IEF Cell (Bio-Rad) at 20 °C with a total of 35 000 V-h. After IEF, the IPG strips were equilibrated for 15 min in an equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS, containing 2% (w/v) DTT, followed by 15 min in an equilibration buffer containing 2.5% (w/v) iodoacetamide. SDS-PAGE in the second dimension was carried out using 15% separation gel with 5% stacking gel at 30 mA for about 3 h, or until the dye line reached to the end of the gel.

The Coomassie Brilliant Blue stained gels were scanned using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer; Bio-Rad). Spots were detected and quantified with the PDQuest software (ver. 8.0; Bio-Rad), on the basis of their relative volume. The amount of a protein spot was expressed as the volume of that spot, which was defined as the sum of the intensities of all pixels that make up the spot. To compensate for subtle differences in sample loading, gel staining, and destaining, the volume of each spot was normalized as a percentage of the total volume of all the spots present in the gel.[13] After automated detection and matching, manual editing was carried out.

Protein Identification by Protein Sequencing. To analyze N-terminal amino acid sequences, 2-DE separated proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, NY) using a semidry transfer blotter (Nippon Eido, Tokyo, Japan) and detected by CBB staining. The stained protein spots of interest were excised from the PVDF membrane and directly subjected to Edman degradation on a gas-phase protein sequencer (Procise cLC; Applied

Biosystems, San Francisco, CA, USA). Protein identification and database searching followed the procedure described previously.[18]

In-Gel Digestion and Protein Preparation for Mass Spectrometry Analyses. Protein spots of interest were excised manually from CBB stained 2-DE gels and then alkylation and protein digestion with trypsin were performed using a robotic system (DigestPro96; Intavis AG, Koeln, Germany). Briefly, protein spots were destained with 50 mM NH_4HCO_3 for 1 h at 40 °C. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The gel pieces were minced and allowed to dry, then rehydrated in 100 mM NH_4HCO_3 with 1 pM trypsin (Sigma-Aldrich, St. Louis, MO) at 37 °C overnight. The tryptic peptides were extracted from the gel grains with 0.1% trifluoroacetic acid in 50% acetonitrile for three times. The generated peptides were purified using a NuTip C-18 (Glygen, Columbia, MD).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry and Data Analysis. The desalted purified peptides were added to α-cyano-4-hydroxycinamic acid matrix and dried on a plate for analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager-DE RP (Applied Biosystems). The pick list of peptide mass was generated by Perspective-GRAMS/386 (for Microsoft Windows, ver. 3.04) software developed by Galactic Industries Corp. (Salem, NH) which is incorporated with Voyager-DE-RP. The obtained peptide mass spectra were searched using an in-house licensed MASCOT search engine (ver. 2.2.04; Matrix Science, London, U.K.), and compared to the soybean genome sequence database (Glyma0, Annotation ver. 4; 75 778 sequences) which was downloaded from the phytozome database (http://www.phytozome.net). Search parameters used fixed cysteine carbamidomethylation and variable methionine oxidation as modifications, peptide mass tolerance ±0.2 Da, fragments ions 1 Da, 1 missed cleavage, and trypsin was specified as the proteolytic enzyme. Peptides were selected in the 500 to 4000 Da mass ranges. For positive identification, the score result of [−10 × Log (P)] had to be over the significance (>60) threshold level ($p < 0.05$). Four criteria were used to assign a positive match with a known protein.[13] These are as follows: (i) The deviation between the experimental and theoretical peptide masses should be less than 50 ppm. (ii) At least four different predicted peptide masses needed to match the observed masses for an identification to be considered valid. (iii) The coverage of protein sequences by the matching peptides must reach a minimum of 15%. (iv) The score that was obtained from the analysis with Mascot software indicates the probability of a true positive identification and must be at least 60. The positive matches were BLAST searched against the UniPort database (http://www.uniprot.org) and/or NCBI protein database (http://www.ncbi.nlm.nih.gov) for updated annotation and identification of homologous proteins.

NanoLC−MS/MS and Data Analysis. The desalted peptide solutions were directly analyzed by nano liquid chromatography-tandem MS as described previously.[19] Using an Ultimate 3000 nanoLC (Dionex, Germering, Germany), peptides were loaded in 0.1% formic acid onto a 300 ìm ID × 5 mm C_{18} PepMap trap column at a flow rate of 25 μL/min. Elution of the peptides from the trap column and their separation and spraying were done using 0.1% formic acid in acetonitrile at a flow rate of 200 nL/min on a Tip column (NTTC-360/75−3, Nikkyo Technos, Tokyo, Japan) with a spray voltage of 1.8 kV. A nanospray LTQ XL Orbitrap MS (Thermo Fisher Scientific, San Jose, CA) was operated in data-dependent acquisition mode with Xcalibur software (ver. 1.4; Thermo Fisher Scientific). Full scan mass spectra were acquired to cover a scan range of 100−2000 m/z with a resolution of 15000. The three most intense ions at a threshold above 1000 were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35% after accumulation to a target value of 1000. Dynamic exclusion was employed within 30 s to prevent repetitive selection of the peptides.

Tandem mass spectrum DTA files were converted to MGF files using BioWorks software (ver. 3.3.1; Thermo Fisher Scientific). The following parameters were set for creation of the peak lists: parent ions in the mass range with no limitation, one grouping of MS/MS scans and threshold at 100. Precursor ion tolerance was 10 ppm. Data files were searched using the MASCOT search engine against the soybean genome sequence database and the NCBI (viridiplantae) database. To set the parameters for database search, carbamidomethylation of cysteines was considered as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Other parameters for search were peptide mass tolerance 10 ppm, fragment mass tolerance ±0.2 Da and positive precursor peptide charge states of 1, 2, and 3. The instrument setting was specified as "ESI-Trap". Protein hits were validated if the identification was with at least five top ranking peptides with the ions scores over the significance threshold level. Individual ions scores >22 and >32 against soybean and NCBI databases, respectively indicate the probability of a true positive identification or extensive homology ($p < 0.05$). In the case of peptides matching multiple members of a protein family, the presented protein was selected based on the highest score and the highest-ranking member of matching peptides.

Immunoblot Analysis. For immunoblot analysis, protein samples were separated by 15% SDS-PAGE and 2-DE and then transferred onto a PVDF membrane using a semidry electrophoretic aparatus. The blotted membrane was blocked for overnight at 4 °C in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 5% non fat milk (Skim milk; Difco, Sparks, MD). The membrane was subsequently incubated with the polyclonal antibodies anti-HSP70 (H5147, Sigma-Aldrich), anti-chaperonin (CPN)-60 subunit beta (SMC-110 A/B, StressMarq,Victoria, Canada) and anti-chloroplast small HSP (a kind gift from Prof. Byung-Hyun Lee, Gyeongsang National University, Korea) at 1:10000 dilutions for 5 h at room temperature. Secondary antibodies were anti-mouse or anti-rabbit IgG with conjugated horseradish peroxidase (Bio-Rad). After incubation for 1 h with the appropriate HRP-conjugated secondary antibodies, the immunoblot signals were detected using ECL plus Western blotting detection kit (GE Healthcare, Piscataway, NJ) following the manufacturer's protocol and visualized on X-ray films (Hyperfilm, GE Healthcare).

Statistical Analysis. The measured biochemical parameters and spot intensities were statistically analyzed by one-way ANOVA and Least Significant Difference (LSD) test to determine significant differences among group means. Statistical analysis was carried out using the data obtained from three different sets of independent biological samples. $P \leq 0.05$ was considered to be statistically significant. The statistical package SAS,

version 9.1 (SAS/STAT Software for PC, SAS Institute Inc., Cary, NC) was used for all statistical analyses.

Results

2-DE and Selection of Heat-Exposed Sample for Analysis. The primary objective of the present study was to characterize the tissue-specific protein expression profile and to identify tissue-specific proteins in the leaves, stems, and roots of soybean seedlings in response to high-temperature stress. Therefore, to get the maximum number of proteins in gel, the total soluble proteins were extracted from three different tissues such as leaves, stems, and roots of control and heat-treated plants and separated by 2-DE. The present protein extraction method was equally efficient to a wide range of soybean tissues for 2-DE analysis.[13] A broad range of p*I* (3−10) was examined. However, the p*I* 3−10 separation indicated that the majority of the leaf proteins were distributed within the narrower p*I* range of 5−8 (Supplemental Figure 1A, Supporting Information) and that to separate individual proteins higher resolution is needed. Therefore in our further analyses, the 5−8 p*I* range was used for subset proteome analysis (Supplemental Figure 1B, Supporting Information). RuBisCO is the most abundant protein (30−50%) in leaves, and its presence in samples often limits the detection of differentially expressed, low-abundance proteins in 2-DE analysis. A PEG-mediated fractionation was applied to remove the RuBisCO from the leaf samples; this fractionation enriched for many low-abundance proteins allowing for the detection of the proteins in the fractionated samples that were undetectable in the 2-DE gels containing the total soluble protein samples (Supplemental Figure 2, Supporting Information).

To determine the most suitable exposure time for investigation of the maximum number of heat-induced differentially expressed proteins, soybean seedlings were exposed to heat stress (40 ± 2 °C) for 6, 12, or 24 h, and total soluble protein contents of leaf samples were separated by 2-DE (Supplemental Figure 3A, Supporting Information). It is interesting to note that the maximum number of heat-induced differentially expressed proteins was observed after a 12 h heat-stress treatment and after that the abundance of many proteins were declined and spots were less reproducible in gels (Supplemental Figure 3B, Supporting Information). In addition, we found that the leaves were wilted after 24 h of heat-stress treatment (Supplemental Figure 4, Supporting Information), and these plants were probably also subjected to a drought-like stress. Taken together, these results indicated that exposing soybean seedlings to a heat-stress treatment for 24 h or more may not be suitable for a proteome analysis of heat-responsive proteins. Therefore, in the present study, a 12 h heat-stress treatment was used for tissue-specific proteomic analysis.

Heat-Induced Tissue-Specific Proteome Profiling of Soybean Seedlings. The total soluble and PEG-fractionated protein samples of control and heat-treated (12 h) leaves were separated by 2-DE in the p*I* range 5−8. The 2-DE proteome map of total soluble proteins from leaves represented more than 800 spots that were reproducibly detected in each CBB-stained gel of each biological replicate (Supplemental Figure 5, Supporting Information). Quantitative image analysis of three biological replicates using PDQuest software revealed that a total of 43 proteins showed at least 1.5-fold ($p < 0.05$) differences in expression value between the control and heat-treated samples, in which 34 spots were up-regulated and 9 spots were down-regulated in response to the heat treatment. More than 700 protein spots were reproducibly detected in each PEG-fractionated CBB-stained gel (Supplemental Figure 6, Supporting Information); of these spots, 22 exhibited significant differential expression in three biological samples. Of these differentially expressed spots, 10 were up-regulated and 12 were down-regulated following exposure to heat stress. Thus, a total of 65 spots showed a significant (at least 1.5-fold, $p < 0.05$) difference in expression level between control and heat-treated leaf samples.

The 2-DE of stem proteins represented approximately 900 spots (Supplemental Figure 7, Supporting Information), and 35 spots exhibited a significant (at least 1.5-fold, $p < 0.05$) difference in expression level between the control and heat-treated samples. Of the differentially expressed spots, a total of 29 spots were up-regulated, and 6 spots were down regulated in response to heat stress. Although RuBisCO presented in the stem, the amount was quite lower compared to the leaf and there was no RuBisCO in root. Therefore in this study, PEG-fractionation method was only applied for leaf samples. The root proteome map exhibited the highest number, around 900, of protein spots (Supplemental Figure 8, Supporting Information). Among these, 61 spots showed significant differential expression between control and treated samples, and 30 spots were up-regulated, and 31 spots were down-regulated following exposure to heat stress.

Although the proteome maps of each tissue was distinct from the others, a group of low molecular weight proteins showed similar expression patterns in response to heat stress in all three tissues (Figure 1) whereas these groups of proteins were not differentially expressed in the PEG-fractionated samples (Supplemental Figure 6, Supporting Information). Magnified views of these proteins clearly revealed that most of these proteins were highly up-regulated or newly induced upon exposure to heat (Figure 2). It is interesting to note that differentially expressed proteins in leaves and stems were mostly up-regulated. In contrast, around 50% of roots proteins were decreased in abundance in response to heat stress indicating that root proteins are highly sensitive to thermal stress. Thus, a total of 150 heat-induced differentially expressed protein spots were identified with high confidence by protein sequencing, MALDI-TOF MS, and nanoLC−MS/MS analyses. Of these, 54 proteins were indentified from leaf samples (Table 1), 35 proteins from stem (Table 2), and 61 proteins from root samples (Table 3).

Functional Distribution of the Heat-Responsive Proteins in Soybean Seedlings. Identification of heat-induced differentially expressed proteins from leaves, stems, and roots revealed that many of these proteins shared common identity among the tissues examined (Figure 3A). The 150 heat-induced differentially expressed proteins identified represent a total of 81 nonredundant proteins; 10 of these proteins were differentially expressed in all three tissues, whereas 21, 10, and 34 proteins were uniquely differentially expressed in leaves, stem, and roots, respectively (Figure 3A). Leaves and stems shared the highest number of differentially expressed proteins (Figure 3A). The identified heat-induced proteins were categorized into nine functional classes based on their inferred function (Figure 3B). The largest functional category of heat-induced proteins included proteins involved in protein synthesis and assembly (25%), the next largest group included carbon and carbohydrate metabolism proteins (15%), and the third largest group included antioxidant and defense proteins (12%). Approximately 15% of the proteins were of unknown function. Proteins involved in protein synthesis and folding were up-regulated

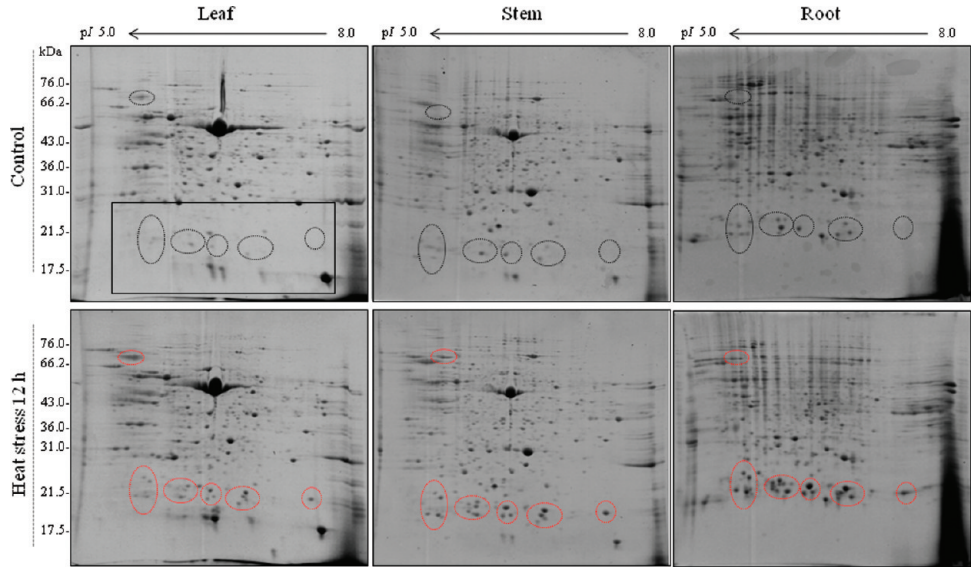

Figure 1. Representative 2-DE gel images of total soluble proteins of control and heat-treated soybean leaves, stems and roots. Plants were grown at 25−20 °C ± 2 °C (day/night) temperatures with 75% relative humidity used as control, whereas plants subjected to 40 °C ± 2 °C for 12 h were used as heat treated plants. Leaves, stems and roots were used as sample for proteomic analysis. Proteins (350 μg) were separated by 2-DE, and visualized with CBB staining. The circled areas indicate proteins that were differentially expressed in a same manner among the tissues under heat stress.

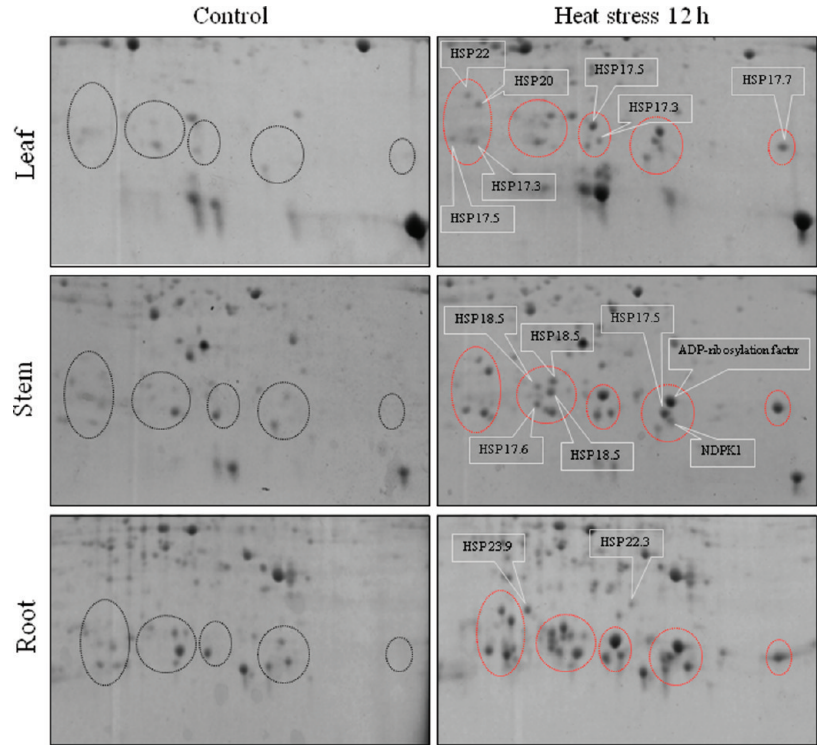

Figure 2. Differential expression of heat-responsive low molecular weight proteins in soybean leaves, stems, and roots. Close up views of the boxed area in Figure 1 shown the similar expression pattern of the identified low molecular weight proteins in control and heat exposed leaves, stems, and roots of soybean seedlings.

in all tissues; however, proteins associated with antioxidant defense and secondary metabolism were predominant in leaves and roots, respectively (Figure 3C). As expected, photosynthesis-related proteins were identified in the leaf and stem samples; no proteins associated with this pathway were identified in the root samples (Figure 3C).

Almost half of the differentially expressed proteins were HSPs; therefore, we focused on tissue-specific HSP expression (Figure 3D). Analysis of the HSPs identified in the three tissues revealed that differential expression of a set of HSPs including HSP70, heat shock cognate (HSC) 70, and several low molecular weight HSPs such as HSP22, HSP18.5, HSP 17.5 was common in all three tissues (Figure 3D). These HSPs were newly induced and/or highly up-regulated in each tissue in response to the heat treatment. However, tissue-specific expression of some HSPs was also observed. For instance, the HSP60-β subunit and

Table 1. List of Heat-Induced Differentially Expressed Identified Proteins in Soybean Leaves

SP[a]	homologous protein name	acc. no[b]	ID method[c]	N-sequences	S[d]	SC[e] (%)	M[f]	id[g] (%)	theo[h] M_w/pI	exp[i] M_w/pI	fold[j]	p-value [k]	FC[l]
					Total soluble proteins								
1	70 kDa heat shock protein	CAA52149	LC−MS/MS	−	670	38	54	100	75.4/5.15	73.0/5.15	>2.50	0.0109	P
2	Stromal 70 kDa heat shock	Q02028	MALDI-TOF	−	108	18	9	91	73.8/5.20	73.0/5.20	>2.27	0.0073	P
3	70 kDa heat shock cognate	AAS57913	LC−MS/MS	−	861	45	52	100	71.5/5.14	68.0/5.30	>1.61	0.0434	P
7	Cpn-60 subunit alpha	P08926	Sequencer	SAKEIAFDQH	−	−	−	80	61.9/5.15	62.0/5.10	>2.19	0.0205	P
8	Cpn-60 subunit alpha	P08926	Sequencer	SAKEIAFDQH	−	−	−	80	61.9/5.15	62.0/5.20	>1.79	0.0226	P
10	Cpn-60 subunit beta	P08927	Sequencer	AKELHFNKDG	−	−	−	100	62.9/5.85	60.0/5.50	>3.45	0.0226	P
11	Cpn-60 subunit beta	P08927	Sequencer	AKELHFNKDG	−	−	−	100	62.9/5.85	60.0/5.60	>1.78	0.0183	P
14	Cpn-60 subunit beta	P08927	LC−MS/MS		582	34	24	100	63.2/5.85	58.0/5.30	>2.22	0.0114	P
17	Elongation factor Tu	CAA46864	MALDI-TOF	−	63	15	5	93	52.2/6.21	48.0/5.50	<0.73	<.0001	P
20	Glutamine synthetase	AAK43833	MALDI-TOF	−	72	23	5	98	47.9/6.42	47.0/5.70	<0.52	0.0146	AM
21	GAPD A subunit	ABA86963	MALDI-TOF	−	66	18	5	99	43.4/7.62	50.0/6.60	>1.66	0.021	C
25	Cysteine synthase	ABO15564	MALDI-TOF	−	64	16	4	99	34.5/5.53	39.0/5.50	<0.35	0.0003	AM
26	Phosphoglycerate kinase	EEF48756	MALDI-TOF	−	63	13	6	93	50.2/7.79	42.0/5.70	<0.56	0.0002	EM
27	Fructose-bisphosphate aldolase	Q01516	Sequencer	SSYADELVKT	−	−	−	100	38.6/5.83	38.0/5.70	<0.55	0.0007	C
49	HSP 20 kDa chloroplastic	O65282	Sequencer	ATVVAPKYTA	−	−	−	100	26.8/8.86	29.0/5.40	>2.97	0.0036	P
53	Carbonic anhydrase	P17067	MALDI-TOF	−	108	31	6	90	28.3/6.10	27.0/6.80	<0.53	<.0001	EM
56	HSP 22 kDa, mitochondrial	Q39818	Sequencer	NTNAMNQYDN	−	−	−	90	23.9/6.34	23.5/5.30	>4.22	0.0044	P
57	22.0 kDa class IV HSP	P30236	Sequencer	SLLPFIDPPT	−	−	−	89	21.9/6.24	22.5/5.35	>15.6	0.0113	P
60	Hsp23.9	AAB03096	Sequencer	STNAMWQYDN	−	−	−	100	23.9/6.34	23.0/5.50	>22.1	0.0109	P
63	Proteasome subunit beta	EEF45263	MALDI-TOF	−	66	26	4	93	22.6/5.86	24.0/5.80	>3.79	0.0369	P
68	17.5 kDa class I HSP	P04794	MALDI-TOF	−	65	23	4	98	17.5/5.98	18.5/6.00	>7.71	0.0007	P
69	17.3 kDa class I HSP	P02519	LC−MS/MS		180	70	28	100	17.3/6.17	17.5/6.10	>60.1	<.0001	P
71	ADP-ribosylation factor	AAD17207	LC−MS/MS	−	370	68	21	100	20.3/6.42	17.5/6.60	>65.3	0.0132	P
72	Small molecular HSP 17.5	ABK92179	LC−MS/MS	−	425	70	14	100	17.9/6.62	17.5/6.60	>248.7	0.0370	P
73	NDPK I	Q39839	LC−MS/MS	−	337	47	30	100	16.4/5.93	17.0/6.65	>26.6	0.0122	A
75	18.5 kDa class I HSP	P05478	LC−MS/MS		166	58	10	81	17.0/6.77	17.5/6.75	>23.6	0.0489	P
76	17.7 kDa class I small HSP	P92309	LC−MS/MS	−	373	79	24	85	17.8/6.85	17.0/6.85	>21.7	0.0010	P
77	RuBisCO small subunit rbcs1	AAG24882	MALDI-TOF	−	171	66	9	99	14.6/6.82	14.5/7.00	<0.56	0.0005	Ph
80	RuBisCO small subunit rbcs1	AAG24882	MALDI-TOF	−	96	37	6	99	14.6/6.82	14.5/6.10	<0.03	<.0001	Ph
81	RuBisCO small subunit rbcs3	AAG24884	MALDI-TOF	−	77	22	5	100	19.3/8.67	14.5/5.80	>81.4	0.0006	Ph
82	RuBisCO small subunit rbcs1	AAG24882	MALDI-TOF	−	179	55	9	99	14.6/6.82	14.5/6.00	<0.61	0.0002	Ph
83	RuBisCO small subunit rbcs1	AAG24882	MALDI-TOF	−	118	52	6	99	14.6/6.82	14.5/6.10	>1.51	0.0054	Ph
84	mRNA-capping enzyme	Q84424	Sequencer	QLLPFANIPS	−	−	−	88	37.8/8.23	20.0/6.20	>2.87	0.0004	P
					PEG-fractionated proteins								
P-5	Cpn-60 subunit alpha	P08823	Sequencer	SAKEIAFDQL	−	−	−	89	57.5/4.83	62.0/5.10	>16.6	0.0875	P
P-6	Cpn-60 subunit alpha	P08823	Sequencer	EAKEIAFDQL	−	−	−	89	57.5/4.83	62.0/5.15	>4.76	0.0099	P
P-8	Unknown	ACU23995	MALDI-TOF	−	64	31	5	91	25.0/6.16	58.0/5.30	<0.43	0.0001	U
P-10	RuBisCO large subunit	BAA19599	MALDI-TOF	−	72	18	6	100	47.9/6.22	52.0/5.90	<0.34	0.0036	Ph
P-11	RuBisCO large subunit	AAV65459	MALDI-TOF	−	94	18	6	100	52.2/6.00	52.0/6.00	<0.28	<.0001	Ph
P-15	Sedoheptulose-1,7-bisphosphatase	EEF31985	Sequencer	EIGDSLEEFL	−	−	−	100	41.9/5.95	40.0/5.20	<0.15	<.0001	C
P-16	Fructose-bisphosphate aldolase	Q01517	Sequencer	GSYADELVKT	−	−	−	100	37.8/5.47	38.0/6.30	<0.86	0.0002	C
P-21	Aldo-keto reductase	EEF32522	MALDI-TOF		77	27	6	77	35.0/6.19	36.0/6.20	>2.68	0.0651	EM
P-22	Ascorbate peroxidase 1	BAC92739	MALDI-TOF		63	33	5	99	27.1/5.63	33.0/5.60	>1.68	0.0033	A
P-23	Stem 31 kDa glycoprotein	P10743	MALDI-TOF		68	25	6	100	29.4/6.72	33.0/6.20	>2.90	0.0228	M
P-25	In2−1 protein	AAG34872	MALDI-TOF		65	34	5	81	27.0/5.29	32.0/5.40	>10.6	0.0207	A
P-26	HSP 20 kDa chloroplastic	O65282	Sequencer	ATVVAPKYTA	−	−	−	100	26.8/8.86	30.0/5.20	>1.82	0.0255	P

Table 1 Continued

SP[a]	homologous protein name	acc. no[b]	ID method[c]	N-sequences	S[d]	SC[e] (%)	M[f]	id[g] (%)	theo[h] M_w/p*I*	exp[i] M_w/p*I*	fold[j]	*p*-value[k]	FC[l]
P-27	Oxygen-evolving enhancer protein 2	P29795	Sequencer	AYGEAANVFG	–	–	–	100	27.7/8.27	29.0/5.30	<0.28	0.0002	Ph
P-28	Triosephosphate isomerase	ABA86966	MALDI-TOF		84	25	6	100	27.4/6.66	31.0/5.90	<0.40	<.0001	EM
P-29	Carbonic anhydrase	AAD27876	MALDI-TOF		63	22	4	93	28.3/6.10	31.0/6.10	<0.06	<.0001	EM
P-30	Carbonic anhydrase	AAD27876	MALDI-TOF		80	31	6	93	28.3/6.10	31.0/6.25	<0.01	<.0001	EM
P-31	Carbonic anhydrase	AAD27876	MALDI-TOF		71	29	5	93	28.3/6.10	31.0/6.30	<0.14	0.0005	EM
P-33	Carbonic anhydrase	AAD27876	MALDI-TOF		80	36	6	93	28.3/6.10	31.0/6.35	<0.34	<.0001	EM
P-36	SOD [Cu−Zn]	EEF38668	Sequencer	ATKKAVAVLK	–	–	–	100	21.5/6.28	21.5/5.20	>4.44	0.0136	A
P-42	Thioredoxin *m*-type	EEF42142	Sequencer	EAQDTAVEVA	–	–	–	100	19.4/9.14	19.0/5.30	>21.1	0.0216	A
P-44	2,4-D inducible GST	AAC18566	MALDI-TOF		61	21	5	99	25.7/6.24	29.0/6.00	>2.96	0.0729	A
P-45	Ferredoxin--NADP reductase	P10933	MALDI-TOF		74	22	7	89	40.6/8.38	39.0/5.90	<0.48	0.0047	C

[a] Spot number in 2-DE gel as shown in Supplementary Figures 5 and 6 (Supporting Information). [b] Accession number of UniPort and/or NCBI database. [c] Protein identification methodology. [d] Mascot Score. [e] Sequence coverage. [f] Number of matched peptides. [g] Identity. [h] Theoretical molecular weight and p*I*. [i] Experimental molecular weight and p*I*. [j] Increased (>) or decreased (<) compared with the control plant. [k] Protein spots showed a significant change in abundance (fold change) by a factor >1.5-fold compared to the control analyzed by LSD test. A *p*-value of ≤0.05 was considered statistically significant. [l] Functional category of the identified proteins. A, antioxidant and defense; AM, amino acid metabolism; C, carbon and carbohydrate metabolism EM; energy and metabolism; M, miscellaneous; P, protein biosynthesis and assembly; Ph, photosynthesis; SM, secondary metabolism; and U, unknown classification.

HSP 22.3 were exclusively induced in leaf and root tissues, respectively. Chloroplast small heat shock protein (ChsHSP) and HSP 60-α subunit were induced only in the leaves and stems, whereas HSP90 and HSP17.6 were overlapped between the stems and roots (Figure 3D).

Tissue-Specific Validation of Some Candidate HSPs by Immunoblot Analysis. Tissue-specific differential expression of some candidate HSPs such as HSP70, HSP 60-β subunit, and ChsHSP were further examined by immunoblot analyses using specific antibodies for each protein (Figure 4). HSP70 was expressed in all tissues, ChsHSP was common between leaf and stem and HSP60-β subunit was exclusively expressed in the leaves, therefore these HPSs were selected for immunoblot analysis. As expected, HSP70 was constitutively expressed and was increased in abundance in response to heat stress in all tissues examined. Immunoblot signal of ChsHSP was strongly detected in leaf and stem with increased expression in the heat-exposed samples and almost undetectable in the root tissue (Figure 4A) suggesting that ChsHSP expression might be restricted to green tissues. On the other hand, the HSP60-β subunit was up-regulated in the heat-treated leaves, and immunblot signals for the HSP60-β were undetectable in stems and roots, consistent with the results of proteomic analysis (Figure 4A). Two spots corresponding to HSP 70 were detected in the proteomic analysis (Tables 1−3), and in the SDS-PAGE immunoblot analysis two cross-reacting polypeptide bands were detected by the anti-ChsHSP antibody. To validate these results additional 2-DE immunoblot analysis was performed; this analysis clearly detected the two HSP70 spots in the approximate molecular mass range of 76.0−66.2 kDa (Figure 4B). Consistent with the SDS-PAGE immunoblot analysis, two spots were also detected in an approximate molecular mass range of 31.0−21.0 kDa in 2-DE immunobloted X-ray film (Figure 4C). Similar result have been reported by Vierling et al.,[20] who also demonstrated two ChsHSPs cross-reacted spots in X-ray film which are a 28 kDa ChsHSP precursor around p*I* 7.0 and a mature ChsHSP (21.0 kDa) in around p*I* 6.0, suggesting that soybean ChsHSP is encoded either by a single gene or two closely linked genes.[20]

Responses of Heat-Induced Low Molecular Weight Proteins to Other Abiotic Stresses. To investigate whether regulation of these low molecular weight proteins (mostly sHSPs) in soybean leaves is particular to heat stress or these proteins also respond to other stresses, soybean seedlings were exposed to cold temperatures (10 °C) and high-oxidant conditions (10 mM H_2O_2) for 12 h. Proteins were extracted from cold- or oxidant-treated leaves, separated by 2-DE and stained by CBB (Figure 5). We found that the candidate low molecular weight proteins that were markedly increased in abundance in response to heat stress were not differentially expressed in response to cold or H_2O_2 stresses (Figure 5). However, a number of proteins other than these HSPs were differentially expressed in response to the cold or H_2O_2 treatments, indicating that either these HSPs are not regulated by cold and/or oxidative stress or the cold and H_2O_2 treatments were insufficient to modulate the expression of these HSPs (Supplemental Figure 9, Supporting Information). Furthermore, immunoblot analysis of HSP70 and ChsHSP revealed that expression of HSP70 was sensitive to both the cold and H_2O_2 treatments; whereas expression of ChsHSP was particular to heat stress (Figure 6). Taken together, these results indicated that the identified soybean sHSPs were particularly sensitive to heat stress and may not be regulated by cold and H_2O_2 stress at least in the given level of treatments.

Table 2. Heat-Induced Differentially Expressed Identified Proteins in Soybean Stems

SP[a]	homologous protein name	acc. no[b]	ID method[c]	S[d]	SC[e] (%)	M[f]	id[g] (%)	theo[h] M_w/pI	exp [i] M_w/pI	fold [j]	p-value [k]	FC [l]
5	Heat shock protein 90−1	ACI31552	LC−MS/MS	804	44	47	100	80.4/4.94	80.0/5.20	>11.1	0.0269	P
6	Heat shock 70 kDa protein	P26413	LC−MS/MS	1715	48	88	100	71.2/5.37	75.0/5.45	>32.7	0.0065	P
7	70 kDa heat shock cognate protein 1	AAS57912	LC−MS/MS	2156	67	112	97	71.4/5.10	72.0/5.30	>3.07	0.0168	P
8	70 kDa heat shock cognate protein 1	AAS57912	LC−MS/MS	1960	70	81	96	71.4/5.11	72.0/5.35	>5.28	0.0377	P
9	60 kDa chaperonin subunit alpha	P08926	LC−MS/MS	3156	82	122	90	61.7/5.29	64.0/5.20	>4.39	0.0280	P
12	UDP-glucose pyrophosphorylase	AAL33919	LC−MS/MS	2717	83	99	92	51.5/5.20	55.0/5.40	>6.07	0.0053	C
13	Glutamine synthetase	AAK43833	LC−MS/MS	787	57	43	98	47.9/6.42	42.0/5.50	<0.43	0.0011	AM
14	Elongation factor Tu, chloroplastic	Q43467	LC−MS/MS	960	65	49	100	52.1/6.21	52.0/5.60	<0.21	<.0001	P
15	Proliferating cell nuclear antigen	O82134	LC−MS/MS	441	54	16	95	29.7/4.68	39.0/5.15	>2.11	0.0085	M
16	Oxygen-evolving enhancer protein 1	P14226	LC−MS/MS	225	61	97	91	35.2/6.66	32.0/5.30	<0.55	<.0001	Ph
17	Alcohol dehydrogenase	EEF28759	LC−MS/MS	1340	78	105	90	41.1/6.32	43.0/6.75	>2.69	0.0017	C
18	Formate dehydrogenase	ACZ74696	LC−MS/MS	1574	80	89	90	41.5/6.87	41.0/6.70	>2.66	0.0197	EM
19	dtdp-glucose 4−6-dehydratase	EEF31020	LC−MS/MS	748	69	44	93	38.7/6.68	38.0/6.65	<0.14	<.0001	C
20	Glyceraldehyde-3-phosphate dehydrogenase	ABC75834	LC−MS/MS	1052	70	52	100	36.8/6.72	37.0/6.80	>4.24	0.0303	C
21	Unknown	ACU22719	LC−MS/MS	1152	56	49	100	30.4/7.63	26.0/5.70	<0.46	0.0029	U
22	Heat shock 22 kDa protein, mitochondrial	Q39818	LC−MS/MS	500	45	24	79	23.7/6.47	23.0/5.30	>5.54	0.0204	P
23	22.0 kDa class IV heat shock protein	P30236	LC−MS/MS	1039	59	72	93	22.3/5.74	22.5/5.35	>3.99	0.0265	P
24	RAB1C	CAA98160	LC−MS/MS	715	75	19	98	22.7/5.52	24.5/5.40	>5.53	0.0005	M
25	Hsp23.9	AAB03096	LC−MS/MS	406	40	17	79	23.7/6.93	23.0/5.50	>1.99	<.0001	P
26	Stem 31 kDa glycoprotein	P10743	LC−MS/MS	437	37	31	100	29.4/6.72	22.0/5.80	<0.38	0.0008	M
27	Small heat shock protein, chloroplastic	P09887	LC−MS/MS	602	61	49	100	26.0/7.74	24.0/6.10	>17.0	0.0011	P
28	17.5 kDa class I heat shock protein	P04793	LC−MS/MS	52	53	16	100	17.5/5.33	18.0/5.30	>3.30	0.0093	P
29	17.3 kDa class I heat shock protein	P02519	LC--MS/MS	624	76	50	93	17.4/5.52	17.5/5.40	>3.66	0.0313	P
30	18.5 kDa class I heat shock protein	P05478	LC−MS/MS	487	47	22	100	18.4/5.82	19.0/5.60	>62.2	0.0070	P
31	18.5 kDa class I heat shock protein	P05478	LC−MS/MS	352	62	30	100	18.4/5.82	19.0/5.70	>11.6	0.0209	P
32	18.5 kDa class I heat shock protein	P05478	LC−MS/MS	350	72	43	100	18.4/5.82	18.5/5.80	>143.5	0.0265	P
33	17.6 kDa class I heat shock protein	P04795	LC−MS/MS	255	66	38	100	17.6/5.69	18.0/5.60	>39.7	0.0084	P
34	22.0 kDa class IV heat shock protein	P30236	LC−MS/MS	533	55	38	100	22.0/6.23	22.0/6.10	>6.48	0.0039	P
35	17.5 kDa class I heat shock protein	P04794	LC−MS/MS	423	59	152	100	17.5/5.98	20.0/6.10	>817.8	<.0001	P
36	17.5 kDa class I heat shock protein	P04794	LC−MS/MS	203	65	35	100	17.5/5.98	18.5/6.20	>90.2	0.0460	P
37	Unknown	ACU16242	LC−MS/MS	374	62	33	100	22.7/8.69	20.0/6.40	>3.68	0.0014	U
38	ADP-ribosylation factor	AAD17207	LC−MS/MS	370	68	21	100	20.3/6.42	17.5/6.60	>44.6	0.0281	P
39	17.5 kDa class I heat shock protein	P04794	LC−MS/MS	531	83	74	100	17.5/5.98	19.5/6.60	>7.83	0.0012	P
40	Unknown	ACU16259	LC−MS/MS	446	88	46	100	17.6/6.24	18.0/6.60	>7.39	0.0288	U
41	17.7 kDa class I small heat shock protein	ABS72445	LC−MS/MS	978	82	64	85	17.8/6.85	18.0/6.90	>578.7	<.0001	P

[a] Spot number in 2-DE gel as shown in Supplementary Figure 7 (Supporting Information). [b] Accession number of UniPort and/or NCBI database. [c] Protein identification methodology. [d] Mascot Score. [e] Sequence coverage. [f] Number of matched peptides. [g] Identity. [h] Theoretical molecular weight and pI. [i] Experimental molecular weight and pI. [j] Increased (>) or decreased (<) compared with the control plant. [k] Protein spots showed a significant change in abundance (fold change) by a factor >1.5-fold compared to the control analyzed by LSD test. A p-value of ≤0.05 was considered statistically significant. [l] Functional category of the identified proteins. A, antioxidant and defense; AM, amino acid metabolism; C, carbon and carbohydrate metabolism EM; energy and metabolism; M, miscellaneous; P, protein biosynthesis and assembly; Ph, photosynthesis; SM, secondary metabolism; and U, unknown classification.

Table 3. Heat-Induced Differentially Expressed Identified Proteins in Soybean Roots

SP[a]	homologous protein name	acc. no[b]	ID method[c]	S[d]	SC[e] (%)	M[f]	id[g] (%)	theo[h] M_w/pI	exp[i] M_w/pI	fold[j]	p-value[k]	FC[l]
1	Seed lipoxygenase	P24095	LC−MS/MS	4657	62	406	100	96.8/5.78	100/5.40	<0.34	0.0001	A
2	Seed lipoxygenase	P24095	LC−MS/MS	7936	71	557	100	96.8/5.78	100/5.45	<0.41	0.0001	A
3	Heat shock protein 90−1	ACI31552	LC−MS/MS	1773	50	191	100	80.7/4.94	82.0/5.15	>4.42	0.0020	P
4	HSC 70 kDa protein 1	AAS57912	LC−MS/MS	4083	46	209	96	71.4/5.17	72.0/5.20	>2.50	0.0261	P
5	Heat shock 70 kDa protein	P26413	LC−MS/MS	4779	50	351	100	71.2/5.37	80.0/5.25	>25.4	0.0065	P
6	Heat shock 70 kDa protein	P26413	LC−MS/MS	5078	57	326	100	71.2/5.37	80.0/5.30	>23.6	0.0285	P
7	Heat shock 70 kDa protein	P26413	LC−MS/MS	6790	57	383	100	71.2/5.37	80.0/5.45	<0.29	<.0001	P
8	Unknown	ACU21101	LC−MS/MS	2283	74	236	100	42.0/5.23	48.0/5.20	<0.42	0.0020	U
9	Actin	AAB40078	LC−MS/MS	1877	75	206	100	42.0/5.23	46.0/5.30	<0.35	<.0001	M
10	Cytosolic glutamine synthetase	AAG24873	LC−MS/MS	927	48	90	100	39.1/5.48	40.0/5.40	<0.35	0.0010	AM
11	Succinyl-coa ligase beta subunit	ABQ10186	LC−MS/MS	1009	24	98	100	45.5/6.41	39.0/5.25	<0.31	0.0018	EM
12	Pyruvate dehydrogenase	EEF50085	LC−MS/MS	1443	36	104	90	38.9/5.70	38.0/5.25	<0.27	<.0001	EM
13	UDP-glucose:protein transglucosylase	AAT44738	LC−MS/MS	369	42	46	93	45.0/6.10	38.0/5.35	<0.47	0.0002	C
14	NADPH:isoflavone reductase	CAA06027	LC−MS/MS	703	45	96	100	35.7/5.30	36.0/5.40	<0.23	<.0001	SM
15	Fructokinase	EEF29025	LC−MS/MS	3960	75	247	86	35.5/5.29	34.0/5.30	<0.41	<.0001	C
16	Beta-ketoacyl-ACP synthetase I	AAF61730	LC−MS/MS	740	16	55	100	50.1/7.01	52.0/6.10	<0.20	<.0001	SM
17	GT4	ACT34899	LC−MS/MS	525	48	71	67	53.2/6.15	50.0/6.15	<0.05	<.0001	C
18	Unknown	ACU20227	LC−MS/MS	1416	26	135	100	41.0/6.20	48.0/6.10	<0.22	<.0001	U
19	Isoflavone reductase homologue 2	AAF17578	LC−MS/MS	1589	60	153	100	33.9/5.60	34.0/5.60	<0.34	0.0002	SM
20	Unknown	ACU23836	LC−MS/MS	870	38	54	100	27.4/5.74	32.0/5.40	<0.16	<.0001	U
21	Lactoylglutathione lyase	ACJ11750	LC−MS/MS	483	41	53	83	31.7/5.62	33.0/5.50	>4.17	0.0057	SM
22	Alcohol-dehydrogenase	AAC97495	LC−MS/MS	1082	58	101	100	37.0/6.13	40.0/6.15	>4.59	0.0002	C
23	Glutamate dehydrogenase 1	CAI53673	LC−MS/MS	1024	38	70	100	44.8/6.04	40.0/6.30	<0.35	0.0001	AM
24	Unknown	ACU19453	LC−MS/MS	396	33	27	100	38.5/6.01	38.0/6.50	<0.26	<.0001	U
25	Unknown	ACU22795	LC−MS/MS	1075	74	106	100	38.0/5.94	35.0/6.20	<0.56	<.0001	U
26	Chalcone reductase	ACH42079	LC−MS/MS	778	60	54	66	35.2/6.13	34.0/6.15	<0.57	<.0001	SM
27	NAD(P)H-dependent 6′-deoxychalcone synthase	P26690	LC−MS/MS	453	76	120	100	35.2/6.32	33.0/6.20	<0.10	<.0001	SM
28	Peroxisomal 3-ketoacyl-CoA thiolase precursor	BAG09380	LC−MS/MS	267	46	35	100	49.2/7.95	42.0/7.30	<0.33	0.0003	M
29	Peroxidase precursor	AAD11482	LC−MS/MS	424	55	77	100	39.1/8.45	39.0/7.10	<0.38	<.0001	A
30	Cytosolic fructose-1,6-bisphosphate aldolase	CAA06308	LC−MS/MS	433	56	77	89	38.5/7.12	38.0/7.10	<0.47	0.0006	C
31	Annexin	CAA75308	LC−MS/MS	442	56	97	84	35.7/6.79	34.0/6.90	<0.46	0.0001	M
32	Unknown	ACU17665	LC−MS/MS	318	53	22	100	31.0/5.04	31.0/5.20	<0.37	0.0003	U
33	Eukaryotic initiation factor 4A-11	Q40465	LC−MS/MS	208	17	8	95	46.9/5.29	31.0/5.25	<0.23	<.0001	P
34	Proteasome subunit beta type-3-A	Q9XI05	LC−MS/MS	481	36	21	93	25.1/5.33	31.0/5.30	<0.27	0.0289	P
35	Proteasome subunit beta type 6,9	EEF34407	LC−MS/MS	562	51	76	88	25.1/5.30	26.0/5.40	<0.34	0.0018	P
36	eukaryotic translation initiation factor iso4E	ABU54805	LC−MS/MS	279	44	16	96	22.9/5.71	27.0/5.80	<0.24	0.0003	P
37	Proteasome subunit beta type	EEF45263	LC−MS/MS	824	77	100	93	22.6/5.86	26.0/5.90	>4.18	0.0133	P
38	HSP 22	CAA30168	LC−MS/MS	220	36	18	93	26.1/6.97	25.0/5.90	>326.8	0.0450	P
39	Small heat shock protein	P09887	LC−MS/MS	31	20	5	100	26.0/7.74	26.0/6.10	>15.2	<.0001	P
40	Small heat shock protein	P09887	LC−MS/MS	65	33	13	100	26.0/7.74	25.0/6.00	>10.7	0.0342	P
41	Heat shock 22 kDa protein, mitochondrial	Q39818	LC−MS/MS	76	40	12	79	23.7/6.47	22.0/5.20	>86.7	0.0293	P
42	Hsp23.9	AAB03096	LC−MS/MS	49	28	9	79	23.7/6.93	23.0/5.40	>21.0	0.0295	P
43	22.0 kDa class IV heat shock protein	P30236	LC−MS/MS	480	55	21	99	22.3/5.74	22.0/5.30	>9.97	0.0010	P
44	17.5 kDa class I heat shock protein	P04793	LC−MS/MS	182	47	23	100	17.5/5.33	19.5/5.25	>3.27	<.0001	P
45	17.3 kDa class I heat shock protein	P02519	LC−MS/MS	432	49	54	93	17.4/5.52	19.5/5.30	>7.20	0.0376	P
46	18.5 kDa class I heat shock protein	P05478	LC−MS/MS	35	28	10	100	18.4/5.82	22.0/5.70	>7.50	0.0241	P
47	18.5 kDa class I heat shock protein	P05478	LC−MS/MS	92	28	8	100	18.4/5.82	21.0/5.60	>320.3	0.0033	P
48	17.6 kDa class I heat shock protein	P04795	LC−MS/MS	256	52	16	100	17.6/5.70	20.0/5.60	>45.2	0.0337	P

Table 3 Continued

SP[a]	homologous protein name	acc. no[b]	ID method[c]	S[d]	SC[e] (%)	M[f]	id[g] (%)	theo[h] M_w/pI	exp [i] M_w/pI	fold [j]	p-value [k]	FC [l]
49	17.5 kDa class I heat shock protein	P04793	LC−MS/MS	252	32	17	100	17.5/5.33	19.5/5.60	>127.8	0.0502	P
50	Unknown	ACU13934	LC−MS/MS	84	52	26	100	17.4/5.83	17.0/5.65	>51.5	<.0001	U
51	18.5 kDa class I heat shock protein	P05478	LC−MS/MS	40	24	6	100	18.4/5.82	20.0/5.70	>25.3	0.0424	P
52	Hsp22.3	AAB03097	LC−MS/MS	340	38	21	100	22.4/5.88	23.0/6.40	>32.3	0.0092	P
53	22.0 kDa class IV heat shock protein	P30236	LC−MS/MS	395	52	22	100	22.0/6.23	22.0/6.30	>2.93	0.0021	P
54	17.5 kDa class I heat shock protein	P04794	LC−MS/MS	501	74	66	100	17.5/5.98	21.0/6.00	>2393	0.0432	P
55	17.3 kDa class I heat shock protein	P02519	LC−MS/MS	332	61	24	100	17.3/6.17	20.0/6.10	>339.3	0.0577	P
56	Unknown	ACU16242	LC−MS/MS	802	71	50	99	22.7/8.69	23.0/6.60	>47.8	0.0036	U
57	Chalcone--flavonone isomerase	Q93XE6	LC−MS/MS	325	54	12	100	23.3/6.23	26.0/6.65	<0.14	0.0001	SM
58	ADP-ribosylation factor	AAD17207	LC−MS/MS	424	53	15	100	20.9/6.92	19.0/6.60	>14.0	0.0100	P
59	Small molecular HSP 17.5	ABK92179	LC−MS/MS	425	70	14	100	17.9/6.62	17.5/6.60	>5.37	0.0050	P
60	17.5 kDa class I HSP	P04793	LC−MS/MS	151	40	10	84	17.0/6.77	19.0/6.75	>78.6	0.0019	P
61	17.7 kDa class I small HSP	ABS72445	LC−MS/MS	412	77	41	85	17.8/6.85	18.5/6.90	>436.1	0.0076	P

[a] Spot number in 2-DE gel as shown in Supplementary Figure 8 (Supporting Information). [b] Accession number of UniPort and/or NCBI database. [c] Protein identification methodology. [d] Mascot Score. [e] Sequence coverage. [f] Number of matched peptides. [g] Identity. [h] Theoretical molecular weight and pI. [i] Experimental molecular weight and pI. [j] Increased (>) or decreased (<) compared with the control plant. [k] Protein spots showed a significant change in abundance (fold change) by a factor >1.5-fold compared to the control analyzed by LSD test. A p-value of ≤0.05 was considered statistically significant. [l] Functional category of the identified proteins. A, antioxidant and defense; AM, amino acid metabolism; C, carbon and carbohydrate metabolism EM; energy and metabolism; M, miscellaneous; P, protein biosynthesis and assembly; SM, secondary metabolism; and U, unknown classification.

Discussion

Soybean is sensitive to high temperature stress compared to other legumes.[15] There have been a small number of plant studies investigating global gene or protein expression profiles in response to heat stress; however, little attention has been paid specifically to soybean protein profiles in responses to heat.[11,21] Our initial 2-DE analysis of the protein profiles in leaves revealed that at 12 h heat treatment resulted in the maximum number of up-regulated protein expression and that heat treatments longer than 12 h resulted in decreased expression of several low molecular weight proteins. This initial analysis indicated that a 12 h heat exposure was the most suitable treatment for analyzing the maximum number of heat-responsive proteins in soybean seedlings. Verling et al.[22] demonstrated that the expression level of a ChsHSP was higher after 12 h of heat treatment than after a 24 h treatment, suggesting that the half-life of many proteins including low molecular weight HSPs in soybean at least 12 h under severe heat stress.[23] Immunoblot analysis of the temporal expression of a group of low molecular weight soybean HSPs (15 to 18-kDa) revealed that the sHSPs exhibited higher accumulations during 4 to 16 h heat treatments and after 16 h, sHSPs expression were declined.[24] Therefore, the selection of a 12 h heat treatment for tissue-specific proteomic analysis to investigate the initial protein expression responses of soybean plants to heat stress was realistic.

Activation of a Common Defense Mechanism in Leaves, Stems, and Roots of Soybean under Heat Stress. Although three tissues had distinct responses to heat treatment at the level of protein expression, a group of proteins, mostly HSPs, exhibited similar qualitative and quantitative changes in all three tissues in response to heat stress (Figure 2). Two high molecular weight HSPs and several low molecular weight HSPs represented by several isoforms were common to all three tissues and showed a similar pattern of up-regulation in response to heat stress (Figure 3). It has been reported that a group of HSPs were newly synthesized and normal protein synthesis was impaired when the germinating soybean seedlings were transferred from normal temperature to heat stress.[25]

In the present study, HSP70 was constitutively expressed in all tissues examined; however, its expression level increased at least 2-fold in response to the heat treatment (Figure 4). Consistent with our results, the levels of HSP70 mRNA and protein increases in plants, including soybean, in response to heat stress.[4−6] It has been demonstrated that presence of HSP 70 is needed for normal repression of the heat shock response,[26] and yeast cells missing two HSP70 genes constitutively synthesize a third HSP70 and other HSPs.[27] Moreover, there is strong evidence that transgenic plants that overexpress HSP70/HSC70 showed enhanced tolerance to heat stress.[28] A dual role was suggested for HSP70 in plants: a protective role in thermotolerance and a regulatory effect on heat shock factor activity and hence the autoregulation of the heat shock response.[29] Taken together these results suggest that the HSP70 family may function in regulating other HSPs in plants and be directly involved in thermotolerance of plants.

sHSPs are the most abundant proteins produced under heat stress in higher plants.[25] Hsieh et al.[24] reported that sHSPs accumulate to represent as much as 1% of the total protein content of soybean under heat stress. We also found that among the differentially expressed proteins in each tissue almost one-third are sHSPs (Tables 1−3), and most of these

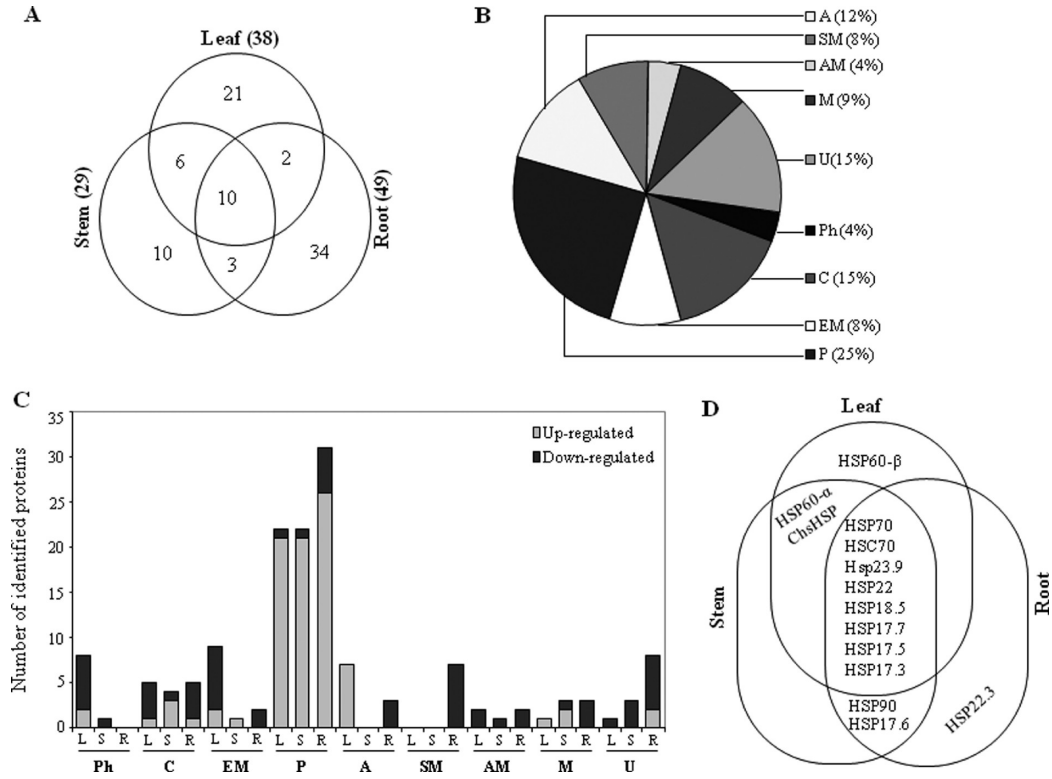

Figure 3. Functional distribution of the heat responsive proteins identified from the leaves, stems, and roots of soybean seedlings. (A) Venn diagram analysis of the heat responsive nonredundant proteins in different tissues analyzed. (B) Functional distribution of the total nonredundant proteins identified in leaves, stems and roots of soybean. (C) Functional classification of the up and down-regulated proteins among the tissues. The black and gray bars indicate heat-induced down- and up-regulated proteins, respectively. (D) Venn diagram illustrating the expression of nonredundant HSPs under heat stress in different tissues of soybean seedlings. L, leaf; S, stem; R, root; A, antioxidant and defense; AM, amino acid biosynthesis and metabolism; Ph, photosynthesis; EM, energy metabolism; P, protein synthesis and assembly; SM, secondary metabolism; M, miscellaneous; and U, unknown function.

were common to all three tissues (Figure 3). These sHSPs were either newly synthesized or markedly up-regulated in response to heat stress. Nuclear-encoded plant sHSPs are targeted to different subcellular compartments, that is, cytosol, endoplasmatic reticulum (ER), chloroplast, peroxisome, and mitochondria,[30,31] and show considerable heterogenecity in isoelectric points, molecular weight, stability, and radiolabel incorporation.[25] In this study, the sHSPs that were common to all three tissues localize to different subcellular compartments including the cytosol (HSP18.5, HSP17.7, HSP17.5, and HSP17.3), the ER (HSP22.3 and HSP22), and the mitochondria (HSP22). However, in each tissue, several spots were identified with same homology of HSPs. Similar phenomena are frequently observed in gel-based proteomic studies.[32,33] Some of the possible reasons for protein shifting in 2-DE gels and identification of the same proteins in different spots are as follows: post-translational modifications; isoforms with different signal or targeting sequences; presence of multimeric forms of the proteins; and translated gene products from different paralogs of a multigene family.[33] In soybean, the diversity of sHSPs proteins can be attributed, at least in part, to their derivation from multigene families.[34] Moreover, molecular chaperones, such as HSPs, are common targets of post-translational modification in plants during normal development or under stress conditions.[35] Therefore, in the present study identification of the same protein in multiple spots was not unusual.

A genome-wide comparative sequence analysis of sHSPs between three model plants revealed that more than 50% of the sHSPs were localized in the cytosol, and these proteins are highly diversified and could be grouped under six subclasses (I−IV).[31] The results of our analysis of the response of heat-induced sHSPs to cold or H_2O_2 stresses were both similar and dissimilar to earlier published results of *Arabidopsis* sHSPs.[31] For instance, 18.5 sHSPs was induced by heat stress in all tissues; however its expression did not change in response to cold or H_2O_2 stress. This result was consistent with results on *Arabidopsis* 18.5 sHSP expression, which increases 2-fold in response to heat stress and does not change in response to other stresses such as salt, anoxia, osmotic, and oxidative stresses.[31] In *Arabidopsis*, cytosolic sHSPs (17.5 and 17.6 kDa), mitochondrial sHSPs, and ER sHSPs (22.0 and 22.3 kDa) were differentially expressed in response to a wide range of abiotic stresses, including oxidative stress.[31] In contrast, the differentially expressed sHSPs identified in this study did not changes there expression significantly in response to cold or H_2O_2 treatments (Figure 5). In the present study, a set of sHSPs were induced and expressed in a same manner in all three tissues in response to heat treatment, indicating that these sHSPs play a critical role in cells by preventing irreversible protein aggregation/insolubilization and maintaining denatured proteins in a folding-competent state under heat stress conditions thus maintain the thermo-stability in each tissue.

Regular Amino Acid and Protein Biosynthesis Mechanisms are Impaired During Heat Stress. The expression of a number of other proteins (in addition to the sHSPs) involved in amino acid and protein biosynthesis also changed in a same manner in leaves, stems, and roots subjected to the heat treatment. For instance, glutamine synthase (GS) was heavily

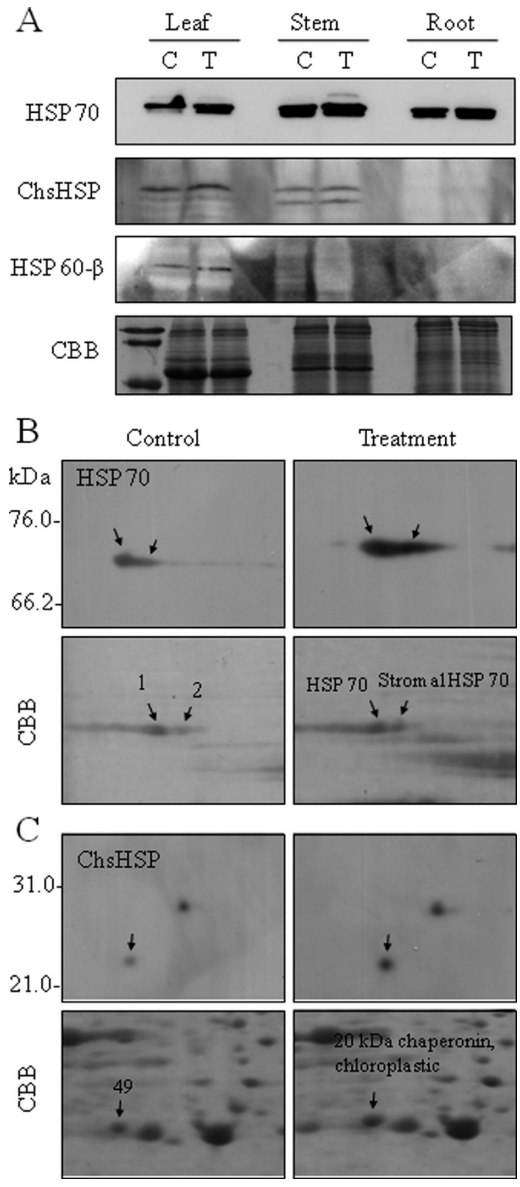

Figure 4. Immunoblot analysis of several candidate HSPs in response to heat stress. (A) Proteins (25 μg) were extracted from control (C) and heat-treated (T) leaves, stems and roots, and separated by SDS-PAGE, followed by electrotransfer to PVDF membranes. Immunodetection was performed with anti- HSP70, ChsHSP and CPN-60 β antibodies. (B and C) 2-DE immunodetection of HSP70 and ChsHSP in control and heat exposed leaf samples. Proteins (350 μg) were extracted and separated by the 2-DE, followed by electrotransfer to PVDF membranes. Immunodetection was performed with specific antibodies as described above. Arrows indicate the HSP70 and ChsHSP. CBB stained proteins are shown in the lower panel to verify the equal loading control.

down-regulated in all three tissues, while cysteine synthase (CS) and glutamate dehydrogenase (GDH) were down-regulated in the heat-stressed leaves and roots, respectively (Tables 1−3). Assimilation of ammonium is a critical biochemical process for plant growth and development, and it involves two enzymatic pathways wherein GS catalyzes the assimilation of ammonium to glutamine using glutamic acid as its substrate. Together with GS, a number of other enzymes such as GDH also play a key role in maintaining the carbon/nitrogen balance within plant cells.[36] CS is the key enzyme in cysteine biosynthesis pathway, and cysteine is used as a precursor in synthesis of glutathione, which involved in heat stress tolerance in plants.[37] GS, GDH, and/or other proteins associated with amino acid biosynthesis are down-regulated in plants subjected to heat stress.[8,10] Moreover, photosynthetic capacity is closely associated with leaf nitrogen,[38] and extreme high temperatures reduce leaf nitrogen and depress nitrogen availability.[39] Furthermore, it has been demonstrated that high temperatures combined with drought lead to a decline in the accumulation of free amino acids in plants.[40] Taken together these results suggest that reduced activity of amino-acid-biosynthesis-related proteins resulted in less osmotic compound production, possibly resulting in stress sensitivity.[40]

A group of proteins associated in protein biosynthesis pathways such as elongation factor-Tu (EF-Tu), mRNA-capping enzyme, eukaryotic translation initiation factor 4A-11, and eukaryotic translation initiation factor iso4E were down-regulated in response to the heat treatment. Under heat stress conditions, the expression of EF-Tu genes was differentially regulated in heat-tolerant and heat-sensitive maize lines, and the relative levels of EF-Tu, showed a positive correlation with the ability to tolerate stress.[41] Eukaryotic translation initiation factors are abundant cytosolic proteins conserved in all eukaryotes and commonly decreased in response to heat stress.[8] Thus, it could be speculated that suppressed expression of the normal complement of cellular proteins under heat stress[30] is one cause of the down-regulation of several proteins associated with protein biosynthesis mechanisms.

The modulation of the heat stress response is dependent on the cellular control of protein degradation and the maintenance of the quality of proteome by the ubiquitin- proteasome system.[42] In the present study, proteasome subunits such as proteasome subunit beta type-3-A, proteasome subunit beta type 6,9, and proteasome subunit beta type were differentially expressed in all tissues under heat stress (Tables 1−3). Our results were very similar to those from an earlier study of Lee et al.[4] who also identified a number of proteasome subunits that were differentially expressed in heat-stressed rice leaves. High temperature is among the major stresses that stimulate protein degradation and lead to tissue senescence or death.[43]

Tissue-Specific Defense and Thermo-Adaptive Mechanisms under Heat Stress. Synthesis of HSPs is thought to be the key mechanism in thermo-tolerance of plants; however, genome-wide transcriptome analysis of model plants demonstrates that several pathways beyond the induction of HSPs are involved in the acquisition of thermotolerance in plants.[44] Proteomic studies of Norway spruce needles and roots[8] and *Agrostis* roots and leaves[9,10] revealed that a large number of proteins (other than some HSPs) were differentially expressed, and the expression of these proteins varied between the tissues. Results of the present study were very consistent with the earlier proteomic studies; however, our results demonstrated significant variability among the tissues for a large number of heat-regulated proteins (Figure 3).

We found that proteins involved in redox homeostasis were mostly regulated in leaves and were up-regulated upon heat stress (Figure 3 and Table 1). A total of six unique antioxidant proteins were significantly up-regulated in response to heat stress (Table 1). Temperature shifts accelerate the production of ROS, such as superoxide (O_2^-), H_2O_2, and hydroxyl radicals.[4,45] In plants, SOD [Cu−Zn] and APX are enzymes in the ROS detoxifying process. In the present study, both SOD [Cu−Zn] and cytosolic APX1 increased significantly following exposure

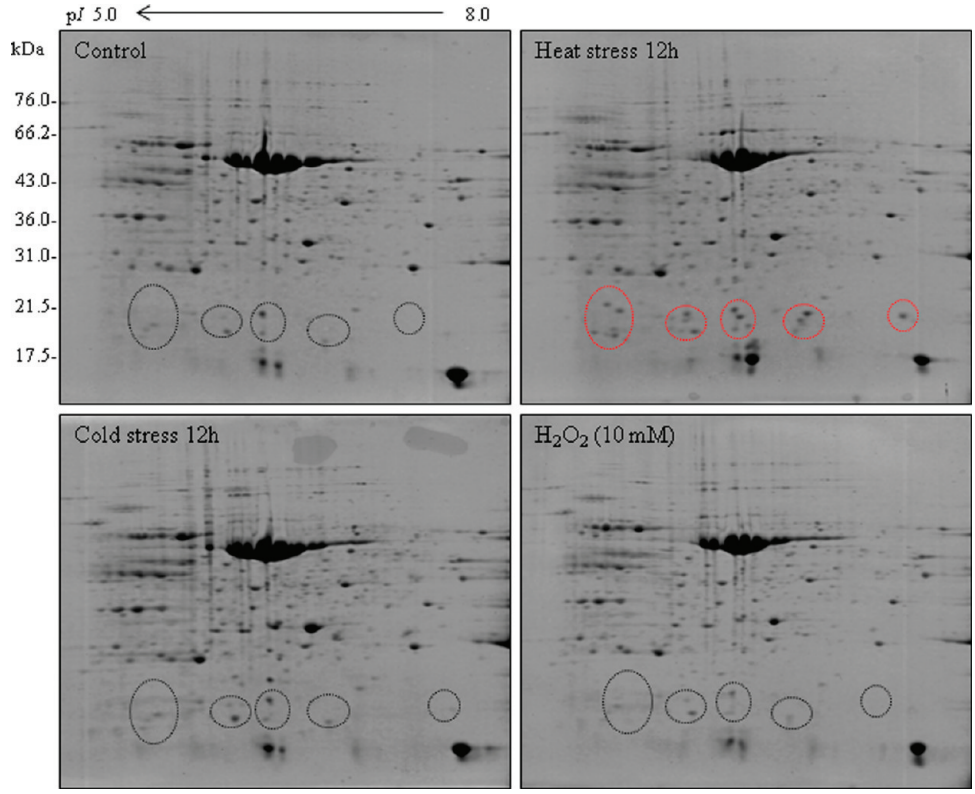

Figure 5. Comparative 2-DE gel images of total soluble proteins of heat, cold and H_2O_2-treated soybean leaves. Proteins (350 μg) were separated by 2-DE, and visualized with CBB staining. The circled areas indicate the low molecular proteins that were differentially expressed in response to heat stress; however did not change in abundance to cold and H_2O_2 treatments.

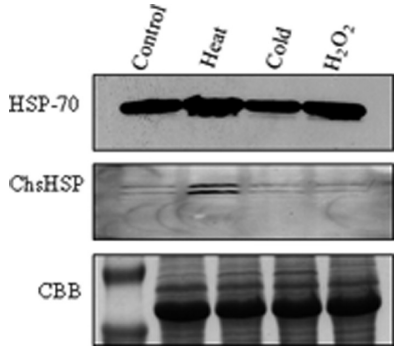

Figure 6. Immunoblot analysis of HSP70 and ChsHSP in soybean leaf proteins in response to other stress conditions. Proteins (25 μg) were extracted from control, heat, cold and H_2O_2-treated soybean leaves, and separated by SDS-PAGE, followed by electrotransfer to PVDF membranes. Immunodetection was performed with anti- HSP70 and ChsHSP antibodies. The signal was detected by chemiluminescence. CBB stained proteins are shown in the lower panel to verify the equal loading control.

to heat, indicating that these two antioxidant enzymes are the first line defense against the heat stress-induced ROS in soybean leaves. A recent genome-wide transcriptome analysis coupled with phenotype analysis of T-DNA insertion mutants of *Arabidopsis* revealed that, together with heat shock transcription factors, a cytosolic APX is a key gene in heat acclimation.[44] The expression of NDPK1 is also known to be induced in other plants by several environmental stresses, including heat shock and oxidative stresses.[4,46] Fukamatsu et al.[47] reported that NDPK1 can interact with other antioxidant enzymes and provided a valuable clue for understanding ROS signaling in plants. We identified an *m*-type thioredoxin that was more than 2-fold increased upon heat stress. It has been confirmed that this protein localizes to the chloroplast (http://urgi.versailles. inra.fr/predotar/predotar.html). It has also been shown that disruption of the *thioredoxin m* gene in cyanobacteria is lethal,[48] and *Ostrxm* RNAi in plants resulted in reduced plant growth and abnormal chloroplast structure.[49] These results indicate that thioredoxin-*m* may be involved in scavenging of heat-induced ROS and protection of the chloroplast structure from thermal stress. The up-regulation of GSTs in response to heat stress might be involved in detoxification of ROS-induced toxic products in cells.[4,46] These results suggest that the antioxidant defense mechanisms are more active in leaves than they are in other organs such as stems and roots during heat stress. Similar to our results, tissue/organ-specific protection against heat-induced oxidative stress has been demonstrated in mustard seedling, wherein leaves were better protected than stems and cotyledons.[45]

Photosynthesis-related proteins that are specific to green tissues such as leaf and stem were, for the most parts, down-regulated in response to heat stress. Proteins associated with primary carbon assimilation and the Calvin cycles were also down-regulated, for the most part, in response to heat stress (Tables 1 and 2). In addition, a number of proteins associated with PS I/II and electron transport such as oxygen-evolving enhancer protein 1and ferredoxin-NADP reductase were also down-regulated following exposure to heat (Table 1). Differential expression of photosynthetic and carbon-assimilation-associated proteins in response to heat stress has been observed previously in poplar and rice.[4,7] Temperatures over 40 °C for prolonged periods (over 2 h) significantly inhibited the activity and quantity of RuBisCO subunits.[50] In leaves, 6 proteins, including the RuBisCO subunits and carbonic anhy-

drase (CA), were down-regulated. CA activity and expression is mostly observed in photosynthetic tissues and is not found in roots.[51] Many physiological studies have demonstrated that short and long-term exposure to extreme temperatures in temperate and tropical crops reduced the net photosynthesis rate and the PSII activity.[15,52] The results taken together are consistent with the hypothesis that the initial decline in photosynthesis-associated proteins may be the cause of reduced CO_2 fixation and net photosynthesis, thus ultimately reducing the products of the Calvin cycle.

The second largest group of proteins that were differentially expressed in roots in response to heat treatments is the secondary metabolite-associated proteins, and most them were down-regulated in the heat-treated plants compared to the control plants (Table 3). Mori et al.[53] reported that temperatures over than 35 °C are an important factor that affect anthocyanin biosynthesis in plants. Moreover, it has demonstrated that gene expressions of the enzymes involved in anthocyanin biosynthesis were affected by high temperature[53] and that the total anthocyanin content was significantly reduced under heat stress. Also consistent with our results, earlier studies in rice[5] and *Agrostis*[9] demonstrated that several proteins associated with flavonoid biosynthesis pathways and lignin synthesis, such as peroxidase, were down-regulated under heat stress. Taken together, these results indicate that the secondary metabolite synthesis pathways, and particularly the flavonoid pathway, are targets of adaptation to heat stress in roots, possibly altering the growth and development of roots.

Although a group of HSPs were shared a common expression pattern among the tissues examined, some of the HSPs showed tissue-specific expression. CPN-60 α and β, HSP 90, ChsHSP, HSP22.3, and HSP17.6 showed tissue-specific responses upon heat treatment (Figure 3). In this study, CPN-60 β was exclusively expressed in heat-treated leaves, and subunit α was common between leaf and stem, but less or no expression was observed in root (Figure 4A). We have previously demonstrated that CPN-60 activity is most abundant in the developing green tissues[13] and might be involved in chloroplast biogenesis[32] and plastid division.[54] Moreover, Salvucci[55] demonstrated that CPN-60 β plays a role in acclimating photosynthesis to heat stress, possibly by protecting rubisco activase from thermal denaturation. Together with CPN-60 β, ChsHSPs was also particular expressed in green tissues (Figure 4). Comparative analysis of ChsHSPs response to cold and H_2O_2 stress indicated that the differential expression of ChsHSP identified in this study may be specific to heat stress (Figure 6). Taken together these results suggest that HSP60 and ChsHSPs may protect the chloroplast proteins from heat stress induced thermal aggregation and denaturation. HSP90−1 was significantly up-regulated in both stem and root tissues. It has been found that *AtHsp90−1* was most prominent in the cotyledons and in a restricted area at the root tip; however, it was barely detectable in the upper meristematic region.[56,57] In roots, a 22.3 kDa HSP was markedly up-regulated under heat stress; however, its expression did not change in response to the cold or H_2O_2 treatment. We found that this sHSP was localized to ER. ER localized sHSP has recently been identified in several plant species such as rice[58] and *Arabidopsis*.[31] Expression of two rice ER sHSPs (22.3 and 21.8 kDa) was markedly up-regulated in roots and shoots under heat stress; however, no expression was observed in response to cold, osmotic, or dehydration stress.[58] In contrast, *Arabidopsis* ER-localized sHSP responds to a wide range of abiotic stresses including heat; however it is not expressed in response to biotic stresses.[31] Taken together, these results indicate that there are differences in the way a given tissue is able to adapt to extreme temperatures.[59]

Concluding Remarks. To our knowledge, this is the first report focusing soybean tissue-specific proteome responses to heat stress. Comparative tissue-specific proteomic analysis indicated that molecular mechanisms that contribute to thermotolerance in soybean seedlings are complex. The present study indicated that a common defense mechanism, associated with the induction of several HSPs, is deployed in leaves, stems, and roots subjected to heat stress. Furthermore, significant up-regulation of some tissue-specific HSPs such as chaperonin 60 and ChsHSPs in leaves and stems suggested that these HSPs protect chloroplast proteins in green tissues from thermal stress. Down-regulation of proteins associated with amino acid biosynthesis and protein translation mechanism (e.g., EF-Tu, and eukaryotic initiation factors) in all three tissues indicated that normal protein synthesis mechanisms are impaired under heat stress. Several proteins involved in distinct pathways were also specifically regulated in individual tissues. For instance, photosynthetic and carbon- assimilation-associated proteins were mostly down-regulated in leaves and stems, and a number of proteins involved in antioxidant defense mechanisms were specifically up-regulated in leaves. In contrast, proteins related to secondary metabolism were mostly down-regulated in roots in response to heat treatment. Taken together, the results from the present study indicated that plants employ a common defense mechanism in leaves, stems, and roots under heat stress, and that each tissue has one or more specific defense and adaptive mechanisms that are probably equally crucial for thermotolerance.

Abbreviations: APX, ascorbate peroxidase; HSP, heat shock protein; PEG, polyethylene glycol; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SOD, superoxide dismutase.

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Supporting Information Available: Supplemental Figure 1. 2-DE gel images of leaf proteins in different p*I* ranges. Proteins (350 μg) were separated by 2-DE and visualized with CBB staining. Supplemental Figure 2. Comparative 2-DE gel images of total soluble and PEG-fractionated proteins of leaves. Proteins (350 μg) were separated by 2-DE and visualized with CBB staining. Supplemental Figure 3. Comparative 2-DE gel images of total soluble proteins of leaves exposed to different heat treatments. Proteins (350 μg) were separated by 2-DE and visualized with CBB staining. Supplemental Figure 4. Morphological views of control and 24 h heat-exposed soybean seedlings. Supplemental Figure 5. Representative 2-DE gel images of three biological replicates of total soluble proteins of control and heat-treated soybean leaf samples. Proteins (350 μg) were separated by 2-DE and visualized with CBB staining. The arrows and circled areas indicate proteins that were differentially expressed in control and treated samples. Supplemental Figure 6. Representative 2-DE gel images of PEG-

fractionated proteins of control and heat-treated soybean leaf samples. Proteins (350 μg) were separated by 2-DE and visualized with CBB staining (A). The arrows indicate proteins that were differentially expressed in control and treated samples. Close up views of the boxed areas are highlighted (B). Supplemental Figure 7. Representative 2-DE gel images of three biological replicates of total soluble proteins of control and heat-treated soybean stems. Proteins (350 μg) were separated by 2-DE, and visualized with CBB staining. The arrows indicate proteins that were differentially expressed in control and treated samples. Supplemental Figure 8. Representative 2-DE gel images of three biological replicates of total soluble proteins of control and heat-treated soybean roots. Proteins (350 μg) were separated by 2-DE, and visualized with CBB staining. The arrows indicate proteins that were differentially expressed in control and treated samples. Supplemental Figure 9. Close up views of some differentially expressed proteins in heat, cold and H_2O_2 treated leaf samples. The arrows indicate proteins that were differentially expressed among the treatments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Schlenker, W.; Roberts, M. J. Nonlinear temperature effects indicate severe damages to US crop yields under climate change. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 15594–15598.

(2) Lobell, D. B.; Burke, M. B.; Tebaldi, C.; Mastrandrea, M. D.; Falcon, W. P.; Naylor, R. L. Prioritizing climate change adaptation needs for food security in 2030. *Science* **2008**, *319*, 607–610.

(3) Penfield, S. Temperature perception and signal transduction in plants. *New Phytol.* **2008**, *179*, 615–628.

(4) Lee, D. G.; Ahsan, N.; Lee, S. H.; Kang, K. Y.; Bahk, J. D.; Lee, I. J.; Lee, B.-H. A proteomic approach in analyzing heat-responsive proteins in rice leaves. *Proteomics* **2007**, *7*, 3369–3383.

(5) Han, F.; Chen, H.; Li, X. J.; Yang, M. F.; Liu, G. S.; Shen, S. H. A comparative proteomic analysis of rice seedlings under various high-temperature stresses. *Biochim. Biophys. Acta* **2009**, *1794*, 1625–1634.

(6) Scafaro, A. P.; Haynes, P. A.; Atwell, B. J. Physiological and molecular changes in *Oryza meridionalis* Ng., a heat-tolerant species of wild rice. *J. Exp. Bot.* **2010**, *61*, 191–202.

(7) Ferreira, S.; Hjerno, K.; Larsen, M.; Wingsle, G.; Larsen, P.; Fey, S.; Roepstorff, P.; Salom, M. Proteome profiling of *Populus euphratica* Oliv. upon heat stress. *Ann. Bot.* **2006**, *98*, 361–377.

(8) Valcu, C. M.; Lalanne, C.; Plomion, C.; Schlink, K. Heat induced changes in protein expression profiles of Norway spruce (*Picea abies*) ecotypes from different elevations. *Proteomics* **2008**, *8*, 4287–4302.

(9) Xu, C.; Huang, B. Root proteomic responses to heat stress in two *Agrostis* grass species contrasting in heat tolerance. *J. Exp. Bot.* **2008**, *59*, 4183–4194.

(10) Xu, C.; Huang, B. Differential proteomic response to heat stress in thermal *Agrostis scabra* and heat-sensitive *Agrostis stolonifera*. *Physiol. Plant* **2010**, *139*, 192–204.

(11) Neilson, K. A.; Gammulla, C. G.; Mirzaei, M.; Imin, N.; Haynes, P. A. Proteomic analysis of temperature stress in plants. *Proteomics* **2010**, *10*, 828–845.

(12) Collins, F. S. Contemplating the end of the beginning. *Genome Res.* **2001**, *11*, 641–643.

(13) Ahsan, N.; Komatsu, S. Comparative analyses of the proteomes of leaves and flowers at various stages of development reveal organ-specific functional differentiation of proteins in soybean. *Proteomics* **2009**, *9*, 4889–4907.

(14) Whiteman, S. A.; Nühse, T. S.; Ashford, D. A.; Sanders, D.; Maathuis, F. J. A proteomic and phosphoproteomic analysis of *Oryza sativa* plasma membrane and vacuolar membrane. *Plant J.* **2008**, *56*, 146–156.

(15) Ortiz, C.; Cardemil, L. Heat-shock responses in two leguminous plants: a comparative study. *J. Exp. Bot.* **2001**, *52*, 1711–1719.

(16) Banzet, N.; Richaud, C.; Deveaux, Y.; Kazmaier, M.; Gagnon, J.; Triantaphylidès, C. Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells. *Plant J.* **1998**, *13*, 519–527.

(17) Kim, S. T.; Cho, K. S.; Jang, Y. S.; Kang, K. Y. Two-dimensional electrophoresis of rice proteins by polyethylene glycol fractionation for protein arrays. *Electrophoresis* **2001**, *22*, 2103–2109.

(18) Komatsu, S. Western blotting/Edman sequencing using PVDF membrane. *Methods Mol. Biol.* **2009**, *536*, 163–171.

(19) Komatsu, S.; Yamamoto, R.; Nanjo, Y.; Mikami, Y.; Yunokawa, H.; Sakata, K. A comprehensive analysis of the soybean genes and proteins expressed under flooding stress using transcriptome and proteome techniques. *J. Proteome Res.* **2009**, *8*, 4766–4778.

(20) Vierling, E.; Nagao, R. T.; DeRocher, A. E.; Harris, L. M. A heat shock protein localized to chloroplasts is a member of a eukaryotic superfamily of heat shock proteins. *EMBO J.* **1988**, *7*, 575–581.

(21) Komatsu, S.; Ahsan, N. Soybean proteomics and its application to functional analysis. *J. Proteomics* **2009**, *72*, 325–336.

(22) Vierling, E.; Harris, L. M.; Chen, Q. The major low-molecular weight heat shock protein in chloroplasts shows antigenic conservation among diverse higher plant species. *Mo1. Cell Biol.* **1989**, *9*, 461–468.

(23) DeRocher, A. E.; Helm, K. W.; Lauzon, L. M.; Vierling, E. Expression of a conserved family of cytoplasmic low molecular weight heat shock proteins during heat stress and recovery. *Plant Physiol.* **1991**, *96*, 1038–1047.

(24) Hsieh, M. H.; Chen, J. T.; Jinn, T. L.; Chen, Y. M.; Lin, C. Y. A class of soybean low molecular weight heat shock proteins: immunological study and quantitation. *Plant Physiol.* **1992**, *99*, 1279–1284.

(25) Mansfield, M. A.; Key, J. L. Synthesis of the low molecular weight heat shock proteins in plants. *Plant Physiol.* **1987**, *84*, 1007–1017.

(26) Tilly, K.; McKittrick, N.; Zylicz, M.; Georgopoulos, C. P. The dnaK protein modulates the heat-shock response of *Escherichia coli*. *Cell* **1983**, *34*, 641–646.

(27) Werner-Washburne, M.; Stone, D. E.; Craig, E. A. Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **1987**, *7*, 2568–2577.

(28) Cazalé, A. C.; Clément, M.; Chiarenza, S.; Roncato, M. A.; Pochon, N.; Creff, A.; Marin, E.; Leonhardt, N.; Noël, L. D. Altered expression of cytosolic/nuclear HSC70−1 molecular chaperone affects development and abiotic stress tolerance in *Arabidopsis thaliana*. *J. Exp. Bot.* **2009**, *60*, 2653–2664.

(29) Low, D.; Brandl, K.; Nover, L.; Forreiter, C. Cytosolic heat-stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones in vivo. *Planta* **2000**, *211*, 575–582.

(30) Vierling, E. The roles of heat shock proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 579–620.

(31) Waters, E. R.; Aevermann, B. D.; Sanders-Reed, Z. Comparative analysis of the small heat shock proteins in three angiosperm genomes identifies new subfamilies and reveals diverse evolutionary patterns. *Cell Stress Chaperones* **2008**, *13*, 127–142.

(32) Lonosky, P. M.; Zhang, X.; Honavar, V. G.; Dobbs, D. L.; Fu, A.; Rodermel, S. R. A proteomic analysis of maize chloroplast biogenesis. *Plant Physiol.* **2004**, *134*, 560–574.

(33) Plomion, C.; Lalanne, C.; Claverol, S.; Meddour, H.; Kohler, A.; Bogeat-Triboulot, M. B.; Barre, A.; Le Provost, G.; Dumazet, H.; Jacob, D.; Bastien, C.; Dreyer, E.; de Daruvar, A.; Guehl, J. M.; Schmitter, J. M.; Martin, F.; Bonneu, M. Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. *Proteomics* **2006**, *6*, 6509–6527.

(34) Czarnecka, E.; Gurley, W. B.; Nagao, R. T.; Mosquera, L. A.; Key, J. L. DNA sequence and transcript mapping of a soybean gene encoding a small heat shock protein. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3726–3730.

(35) Grimsrud, P. A.; den, Os, D.; Wenger, C. D.; Schwartz, D.; Sussman, M. R.; Ané, J. M.; Coon, J. Large-scale analysis of protein phosphorylation in *Medicago truncatula* roots. *Plant Physiol.* **2010**, *152*, 19–28.

(36) Miflin, B. J.; Habash, D. Z. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *J. Exp. Bot.* **2002**, *53*, 979–987.

(37) Kocsy, G.; Szalai, G.; Sutka, J.; Páldi, E.; Galiba, G. Heat tolerance together with heat stress-induced changes in glutathione and hydroxymethylglutathione levels is affected by chromosome 5A of wheat. *Plant Sci.* **2004**, *166*, 451–458.

(38) Llorens, L.; Peñuelas, J.; Estiarte, M. Ecophysiological responses of two Mediterranean shrubs, *Erica multiXora* and *Globularia alypum*, to experimentally drier and warmer conditions. *Physiol. Plant* **2003**, *119*, 231–243.

(39) Wollenweber, B.; Porter, J. R.; Schellberg, J. Lack of interaction between extreme high-temperature events at vegetative and reproductive stages in wheat. *J. Agric. Crop Sci.* **2003**, *189*, 142–150.

(40) Xu, Z. Z.; Zhou, G. S. Combined effects of water stress and high temperature on photosynthesis, nitrogen metabolism and lipid peroxidation of a perennial grass *Leymus chinensis*. *Planta* **2006**, *224*, 1080–1090.
(41) Momcilovic, I.; Ristic, Z. Expression of chloroplast protein synthesis elongation factor, EF-Tu, in two lines of maize with contrasting tolerance to heat stress during early stages of plant development. *J. Plant Physiol.* **2007**, *164*, 90–99.
(42) Mathew, A.; Morimoto, R. I. Role of the heat-shock response in the life and death of proteins. *Ann. N.Y. Acad. Sci.* **1998**, *851*, 99–111.
(43) Furguson, D. L.; Guikema, J. A.; Paulsen, G. M. Ubiquitin pool modulation and protein degradation in wheat roots during high temperature stress. *Plant Physiol.* **1990**, *92*, 740–746.
(44) Larkindale, J.; Vierling, E. Core genome responses involved in acclimation to high temperature. *Plant Physiol.* **2008**, *146*, 748–761.
(45) Dat, J. F.; Foyer, C. H.; Scott, I. M. Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. *Plant Physiol.* **1998**, *118*, 1455–1461.
(46) Ahsan, N.; Lee, D. G.; Lee, K. W.; Alam, I.; Lee, S. H.; Bahk, J. D.; Lee, B. H. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. *Plant Physiol. Biochem.* **2008**, *46*, 1062–1070.
(47) Fukamatsu, Y.; Yabe, N.; Hasunuma, K. *Arabidopsis* NDK1 is a component of ROS signaling by interacting with three catalases. *Plant Cell Physiol.* **2003**, *44*, 982–989.
(48) Muller, E. G.; Buchanan, B. B. Thioredoxin is essential for photosyntheticgrowth. The thioredoxin *m* gene of *Anacystis nidulans*. *J. Biol. Chem.* **1989**, *264*, 4008–4014.
(49) Chi, Y. H.; Moon, J. C.; Park, J. H.; Kim, H. S.; Zulfugarov, I. S.; Fanata, W. I.; Jang, H. H.; Lee, J. R.; Lee, Y. M.; Kim, S. T.; Chung, Y. Y.; Lim, C. O.; Kim, J. Y.; Yun, D. J.; Lee, C. H.; Lee, K. O.; Lee, S. Y. Abnormal Chloroplast Development and Growth Inhibition in Rice Thioredoxin *m* Knock-Down Plants. *Plant Physiol.* **2008**, *148*, 808–817.
(50) Bose, A.; Ghosh, B. Effect of heat stress on ribulose 1,5- bisphosphate carboxylase in rice. *Phytochemistry* **1995**, *38*, 1115–1118.
(51) Majeau, N.; Coleman, J. R. Correlation of carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase expression in pea. *Plant Physiol.* **1994**, *104*, 1393–1399.
(52) Al-Khatib, K.; Paulsen, G. M. High temperature effects on photosynthetic processes in temperate and tropical cereals. *Crop Sci.* **1999**, *39*, 119–125.
(53) Mori, K.; Goto- Yamamoto, N.; Kitayama, M.; Hashizume, K. Loss of anthocyanins in red-wine grape under high temperature. *J. Exp. Bot.* **2007**, *58*, 1935–1945.
(54) Suzuki, K.; Nakanishi, H.; Bower, J.; Yoder, D. W.; Osteryoung, K. W.; Miyagishima, S. Y. Plastid chaperonin proteins Cpn60α and Cpn60β are required for plastid division in *Arabidopsis thaliana*. *BMC Plant Biol.* **2009**, *9*, 38.
(55) Salvucci, M. E. Association of Rubisco activase with chaperonin-60beta: a possible mechanism for protecting photosynthesis during heat stress. *J. Exp. Bot.* **2008**, *59*, 1923–1933.
(56) Prassinos, C.; Haralampidis, K.; Milioni, D.; Samakovli, D.; Krambis, K.; Hatzopoulos, P. Complexity of Hsp90 in organelle targeting. *Plant Mol. Biol.* **2008**, *67*, 323–334.
(57) Prasinos, C.; Krampis, K.; Samakovli, D.; Hatzopoulos, P. Tight regulation of expression of two Arabidopsis cytosolic Hsp90 during embryo development. *J. Exp. Bot.* **2005**, *56*, 633–644.
(58) Sarkar, N. K.; Kim, Y. K.; Grover, A. Rice sHsp genes: genomic organization and expression profiling under stress and development. *BMC Genomics* **2009**, *10*, 393.
(59) Cooper, P.; Ho, T. H.; Hauptmann, R. M. Tissue Specificity of the Heat-Shock Response in Maize. *Plant Physiol.* **1984**, *75*, 431–441.

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