Acta Physiol Plant (2013) 35:501–513
DOI 10.1007/s11738-012-1092-x

ORIGINAL PAPER

Identification of proteins regulated by ABA in response to combined drought and heat stress in maize roots

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Received: 22 February 2012 / Revised: 15 August 2012 / Accepted: 29 August 2012 / Published online: 15 September 2012

Abstract We adopted a proteomics approach to identify and analyze the differential expression of maize root proteins associated with abscisic acid (ABA) regulation under combined drought and heat stress. Using mass spectrometry, we identified 22 major proteins that were significantly up-regulated under combined drought and heat stress. These 22 proteins were classified into 6 functional categories: disease/defense (8), metabolism (3), cell growth/division (3), signal transduction (2), transporters (2) and unclassified (4). Our previous reports showed that ABA regulates the expression of several small heat-shock proteins (sHSPs) in maize leaves subjected to the combination of drought and heat stress; however, no sHSPs were identified among the root proteins up-regulated in this study. RT-PCR and western blot analyses were used to identify six known sHSPs. The maize roots were pretreated with 100 μM of ABA, and subsequently, the expression of the 22 up-regulated proteins and 6 sHSPs was examined. 11 proteins were up-regulated in an ABA-dependent manner, 13 proteins were up-regulated in an ABA-independent manner, and 4 proteins were up-regulated but inhibited by ABA. The up-regulated proteins are interesting candidates for further physiological and molecular investigations of combination stress tolerance in maize.

Keywords ABA · Drought stress · Roots · *Zea mays* L. · Proteomics

Abbreviations

ABA	Abscisic acid
APX	Scorbate peroxidase
CBB	Coomassie brilliant blue
2-DE	Two-dimensional electrophoresis
DTT	Dithiothreitol
GRP2	Glycine-rich RNA-binding protein 2
GST	Glutathione *S*-transferase
IEF	Isoelectric focusing
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MS	Mass spectrometry
NDPKs	Nucleoside diphosphate kinases
PMSF	Phenylmethanesulfonyl fluoride
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
p*I*	Isoelectric point
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
OMT	*O*-Methyltransferase
ZmPR10	*Zea mays* pathogenesis-related protein 10

Communicated by J.-H. Liu.

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Introduction

Drought is one of the most important environmental stress factors, limiting plant growth and agricultural productivity worldwide. Moreover, with global climate change, i.e., rising temperature and altered soil moisture, there is potential for long-lasting droughts across the globe in the near future (Overpeck and Cole 2006; Hashiguchi et al. 2010). High temperature is also an important stress factor that reduces crop production worldwide. Various studies have shown that roots are more sensitive to heat stress than the aboveground organs, and high soil temperature is more harmful than high air temperature to whole-plant growth (Xu and Huang 2001; Liu and Huang 2005). Plants have developed several mechanisms to adapt to heat stress, such as maintenance of membrane stability, scavenging of reactive oxygen species (ROS), production of antioxidants, accumulation and adjustment of compatible solutes, and, most important, chaperone signaling (such as heat-shock proteins, HSPs) and transcriptional activation (for review, see Wahid et al. 2007). During the root acclimation response to high temperature, there is a change in the fluidity of the plasma membrane, leakage of ions and amino acids and an increase in the abundance of ATPase and aquaporins (Lindberg et al. 2005; Iglesias-Acostaa et al. 2010).

However, studies concerning the mechanisms of plant root adaptation to abiotic stress are primarily conducted in response to single stress factors, which is inconsistent with the actual field environment. In nature, plants are typically subjected to a combination of various abiotic stresses, e.g., drought in summer is often accompanied by heat stress. Previous research has revealed that the plant response to combined drought and heat stress is unique and cannot be extrapolated from the responses to individual exposure to single stress factors (Xiong et al. 1999; Rizhsky et al. 2004; Hu et al. 2010a, b). There is little knowledge concerning the mechanism underlying the acclimation of maize roots to a combination of drought and heat stress. Maize is predicted to become the world's most important crop, in terms of the human food supply, by 2050 (Pingali 2001). Therefore, knowledge of the response of maize roots to the combination of drought and heat stress is crucial for understanding maize acclimation to abiotic stress in nature.

Almost all stresses induce the production of HSPs. The induction of HSPs is a common phenomenon in higher plants. HSPs in plants are grouped into five classes based on their approximate molecular weight: (1) HSP100, (2) HSP90, (3) HSP70, (4) HSP60 and (5) sHSPs. In plants, sHSPs are further grouped into six classes according to their intracellular localization and sequence relatedness. The unusual abundance and diversity of sHSPs suggests their importance in plants. In addition to heat stress, plant sHSPs are induced under various stress conditions and at certain developmental stages, which suggests that they play an important role in stress tolerance (for reviews, see Sun et al. 2002; Al-Whaibi 2010). Our previous results showed that three sHSPs, namely, sHSP17.2, sHSP17.4 and sHSP26, were expressed in maize leaves when subjected to heat stress alone or in combination with drought stress but were not detected under control conditions or drought stress alone. Our studies also showed that ABA regulated the expression of sHSP17.2, sHSP17.4, and sHSP26 in the leaves of maize plants in response to the same stresses (Hu et al. 2010a).

The plant hormone ABA regulates many stress responses, such as the inhibition of lateral root growth, guard cell closure and stress gene expression (Xiong et al. 2006; Hu et al. 2008). ABA accumulates in shoot and root tissues and is involved in root-to-shoot signaling under stress (for review, see Schachtman and Goodger 2008); however, it is unclear whether the expression of sHSPs and their relationship to ABA in the roots are similar to those in the leaves when maize plants are exposed to the combination of drought and heat stress.

Therefore, the objective of this study was to analyze the expression profiles of protein associated with ABA, and identify sHSPs associated with ABA in maize roots exposed to the combination of drought and heat stress.

Materials and methods

Plant material and stress treatments

The high-yield hybrid maize (*Zea mays* L.) Zhengdan 958 is widely cultivated in China. Maize seeds were washed in distilled water and germinated on moistened filter papers in the dark following surface sterilization for 10 min in 2 % hypochlorite. After seeds germinated, maize seedlings wrapped with cotton wool were placed in the perforated plastic board, and then the plastic board with maize seedlings was grown in trays of sand containing Hoagland's nutrient solution at 400 μmol m^{-2} s^{-1} of photosynthetically active radiation, a 14/10 h (day/night) cycle, a temperature of 28/22 °C (day/night) and a 75 % relative humidity in a light chamber. When the second leaves were fully expanded, the seedlings were subjected to various treatments. When treated, the plastic board with maize seedlings was transferred and placed in tray with water solution or PEG solution. The plastic board was necessary to avoid the evaporation of solution in tray.

Drought stress was imposed by placing the seedlings in a polyethylene glycol (PEG) solution (−0.7 MPa) for 8 h at 28 °C and 40 % relative humidity. Heat stress was applied to the seedlings cultured in a water solution by

raising the temperature from 28 to 42 °C at an interval of 2 °C/h, followed by incubation at 42 °C for 1 h under 40 % relative humidity; the total treatment lasted 8 h. The combined stress was simultaneously imposed with PEG treatment and heat stress. The control seedlings were placed in a water solution and maintained at 28 °C and 75 % relative humidity. Subsequently, the roots of treated and untreated seedlings were sampled, frozen immediately in liquid N_2 and stored at −80 °C until further analysis.

For the ABA induction experiments, after the second leaves were fully expanded, the maize seedlings were cultured in trays with 100 μM ABA solution for 5 h, and then exposed to the stress treatments for 8 h and sampled as described above.

Three biological replicates were performed per treatment with ten plants.

Maize root protein extraction

The maize root samples were homogenized in liquid N_2 using a mortar and pestle, and the soluble protein was extracted using the SDS/phenol extraction protocol as described in Wang et al. (2006).

Two-dimensional electrophoresis (2-DE)

Proteins were analyzed using 2-DE gel electrophoresis as described (Wang et al. 2006). The isoelectrofocusing was performed with an Ettan III system (GE Healthcare, USA) using IPG strips (7 cm; GE Healthcare) pH 4–7. The second dimension was conducted using 12.5 % SDS polyacrylamide gels. The 2-DE gels were stained with colloidal Coomassie brilliant blue (CBB) G-250 (Kang et al. 2002). A digital image of the gels was captured using a calibrated flatbed optical ImageScanner from Amersham Biosciences with LabScan software at 600 dpi and 256 gray scales and true color in the transmissive mode. The scanner was calibrated using a Calibration OD step tablet from Genomic Solutions Ltd. (Huntingdon, UK). The protein profiles were analyzed using the PDQuest software (BioRad, USA).

Protein identification by mass spectrometry

The proteins identified in the stained 2-DE gels were subjected to in-gel digestion with trypsin (Wang et al. 2009). The digested fragments were analyzed on an Ettan MALDI-TOF Pro Mass Spectrometer (GE Healthcare, USA). The ion acceleration voltage was 20 kV. Each spectrum was internally calibrated using the masses of two trypsin autolysis products. The MS and MS/MS spectra obtained were automatically matched to proteins in NCBI databases (search date, May 6, 2010, http://www.ncbi.nlm.nih.gov/) with the Mascot software (v2.2.03, http://www.matrixscience.com/). The following parameters were adopted for database search: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide mass tolerance ±1.2 Da, fragment mass tolerance ±0.9 Da and missed cleavages 2. Searches were performed in the full range of Mr and p*I*. No species restriction was applied. All of the positive protein identification scores were significant ($P < 0.05$, score >60). Functional categorization of identified proteins was performed using annotation in NCBI and UniProtKB/Swiss-Prot (http://www.ebi.ac.uk/swissprot/) databases. The theoretical Mr and p*I* of the identified proteins were predicted at http://www.expasy.ch/tools/pI_tools.html.

Gene expression analysis using semiquantitative RT-PCR

Total RNA was isolated from the roots using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Germany). Approximately 3 μg of total RNA was reverse transcribed into cDNA using the SuperScript II reverse transcriptase (Invitrogen, USA). The cDNA was amplified using PCR with specific primer sequences for each gene, and the PCR conditions are listed in Table 1. The gene-specific primers listed in Table 1 were designed using the PCR primer designing software tool based on cDNA sequences obtained from the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov). To standardize the results, the relative abundance of maize β-actin was also determined and used as the internal standard. Aliquots of the PCR reactions were loaded onto agarose gels and stained with ethidium bromide. The same individual cDNA samples were used as templates for the RT-PCR analysis of the different genes. The amplification program consisted of an initial denaturation step at 94 °C for 5 min followed by 28–32 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. This experiment was repeated at least three times.

Western blot analysis of sHSP26

Maize root samples were homogenized in an SDS-containing buffer (1:3 g/ml, 2 % SDS, 100 mM Tris–HCl, pH 6.8, 20 % sucrose and 2 % DTT) using a mortar and pestle. After centrifugation at 15,000*g* for 10 min at 4 °C, the supernatants were transferred to clean tubes, immediately frozen with liquid N_2, and stored at −80 °C. The protein content was determined according to the Bradford method (1976) using bovine serum albumin as a standard. Extracts containing 15 μg of protein were loaded and subjected to electrophoresis on a 12.5 % SDS-PAGE gel. Subsequently, the proteins were transferred onto a polyvinylidene difluoride membrane and probed with a polyclonal

Table 1 Primer sequences and amplification conditions

Gene	Primer	Sequence (5′–3′)	T_m (°C)	Product size (bp)	Cycles
HSP16.9	Fw	GCAGCGTGTTCGACCCATT	55	620	30
	Rw	CACCGAACAGCGCAAAGTAC			
HSP17.2	Fw	GTTCTGTTTGTTGAGACGCATAG	55	652	28
	Rw	ACCCGCAAGATTGAGATTGT			
HSP17.4	Fw	AAAACTCGACCAACAATGTCGCT	55	705	28
	Rw	ACACTGATACACGACGGATGAGA			
HSP17.5	Fw	AAAGCAGCGACCCGAGAGAT	55	627	30
	Rw	TAGGATGATTGGCGGTAACAAG			
HSP22	Fw	TTCCTCTAGTTCGCGCTCTG	55	547	32
	Rw	TCATCTCCGCCTTGATCTTG			
HSP26	Fw	CAGATGCTGGACACGATGGA	55	337	30
	Rw	CCTTGCTCTTGTCGCACTCAT			
β-Actin	Fw	AAATGACGCAGATTATGTTTGA	55	712	28
	Rw	GCTCGTAGTGGAGTACGAGC			

Fw forward primer, *Rv* reverse primer

antibody raised against the sHSP26 in maize (Hu et al. 2010b). Immunocomplexes were detected using horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit), and the blots were developed using 3,3-diaminobenzidine tetrahydrochloride. To standardize the results, the relative abundance of maize β-actin was also determined and used as the internal standard. This experiment was repeated at least three times.

Statistical analysis

The relative expression of the identified proteins is represented as the mean of three replicates. The means were compared using one-way analysis of variance and Duncan's multiple-range test at a 5 % level of significance.

Results

2-DE analysis of maize root proteins

To investigate the expression patterns of root protein in maize plants exposed to the combination of drought and heat stress, the proteins extracted from the roots were separated using 2-DE. Triplicate gels were obtained from three independent experiments, and the representative gels are illustrated in Fig. 1a–h. The 2-DE analysis of the protein samples showed more than 450 protein spots that were reproducibly detected in each CBB-stained gel (Fig. 1). Among these proteins, compared with the control (Fig. 1a), 22 specific proteins that were up-regulated under the combination of drought and heat stress treatments were selected for identification in maize roots response to drought (Fig. 1b), heat (Fig. 1c) or the combination of drought and heat stress (Fig. 1d). The relative expression of these proteins is shown in Fig. 2.

Identification of combined stress-responsive protein spots

To identify the differentially expressed proteins, the spots were excised from the preparative gels, in-gel digested using trypsin and analyzed via MALDI-TOF MS. In total, 22 proteins were successfully identified using MALDI-TOF MS analysis (Table 2). Each of the 22 spots contained only one protein. Several of the identified proteins were annotated as putative uncharacterized proteins without specific functions in the maize database. With the exception of spots 15 and 18, all of the proteins shared homologues in other genomes at the amino acid level, indicating similar functions. Based on the 15 functional categories established according to Bevan et al. (1998), the 22 identified proteins were classified into seven functional categories: disease/defense, metabolism, cell growth/division, etc. (Fig. 3). The largest functional category contained proteins involved in disease/defense (26.32 %), which were greatly affected upon the combination of drought and heat stress (Fig. 1), suggesting that scavenging or reducing excessive toxic substances might play an important role in protecting cells from damage under combined drought and heat stress.

ABA effects in response to stress

Compared with the control (Fig. 1a), the abundance of spots 1, 6, 8 and 22 was significantly increased under drought (Fig. 1b) and combined drought and heat stress

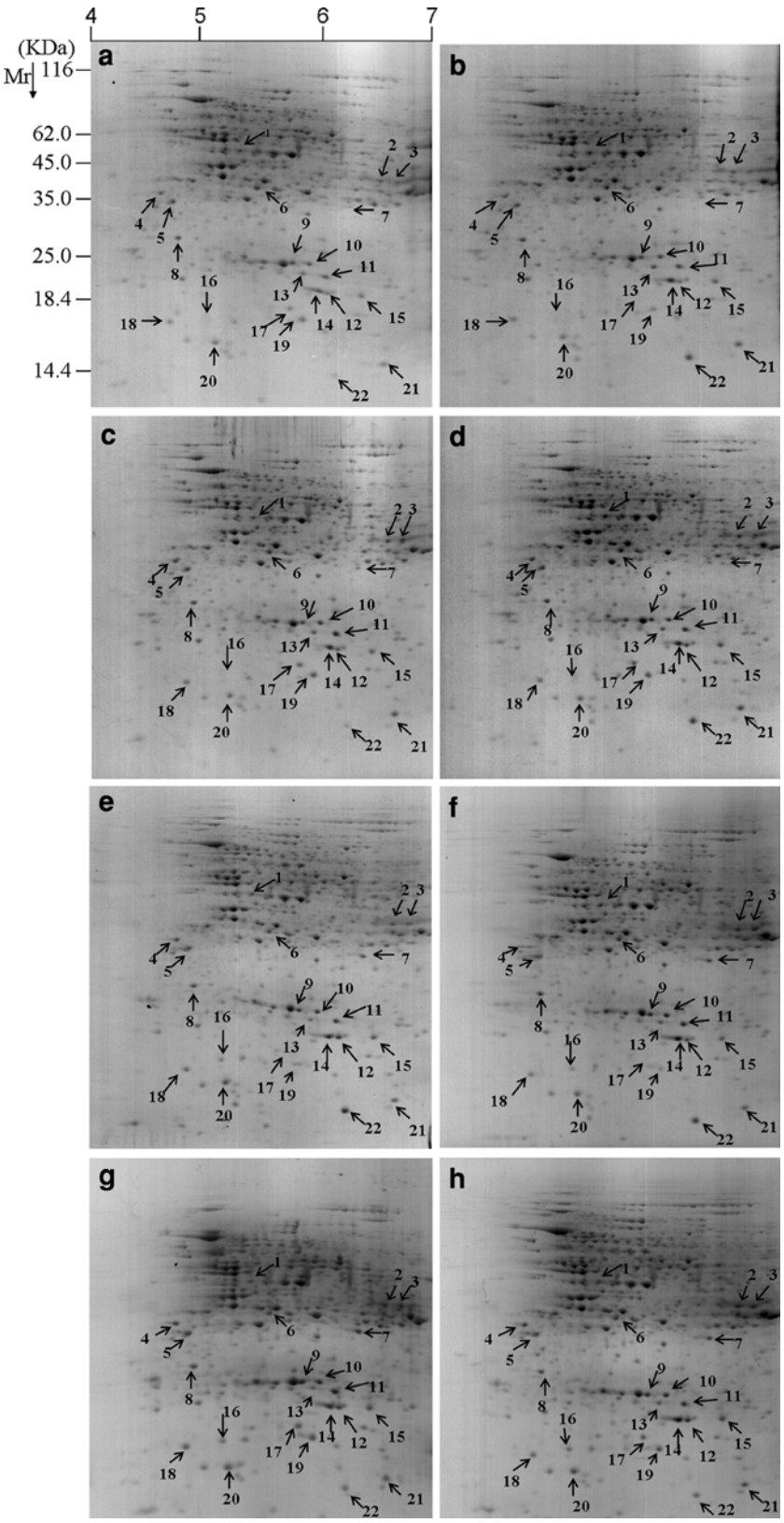

Fig. 1 A 2-DE gel of maize root proteins exposed to drought, heat and their combined stresses and the effects of ABA on maize root proteins exposed to several treatments. After pretreatment with 100 μM ABA for 5 h, the maize plants were exposed to drought, heat and their combined stresses. A total of 800 μg of protein was loaded. The gels were stained with CBB G. Twenty-two root proteins were subjected to MALDI-TOF analysis. **a** Control (distilled water), **b** drought, **c** heat, **d** combined drought and heat stress, **e** 100 μM ABA + distilled water, **f** 100 μM ABA + drought, **g** 100 μM ABA + heat, **h** 100 μM ABA + drought + heat. This figure is a representative result from three biological replicas

(Fig. 1d), but the expression levels of each protein had no difference between two treatments. Pretreatment with 100 μM ABA inhibited the increased expression of spots 1, 6 and 8 under drought (Fig. 1f) and combined drought and heat stress (Fig. 1h), but did not affect the expression of spot 22.

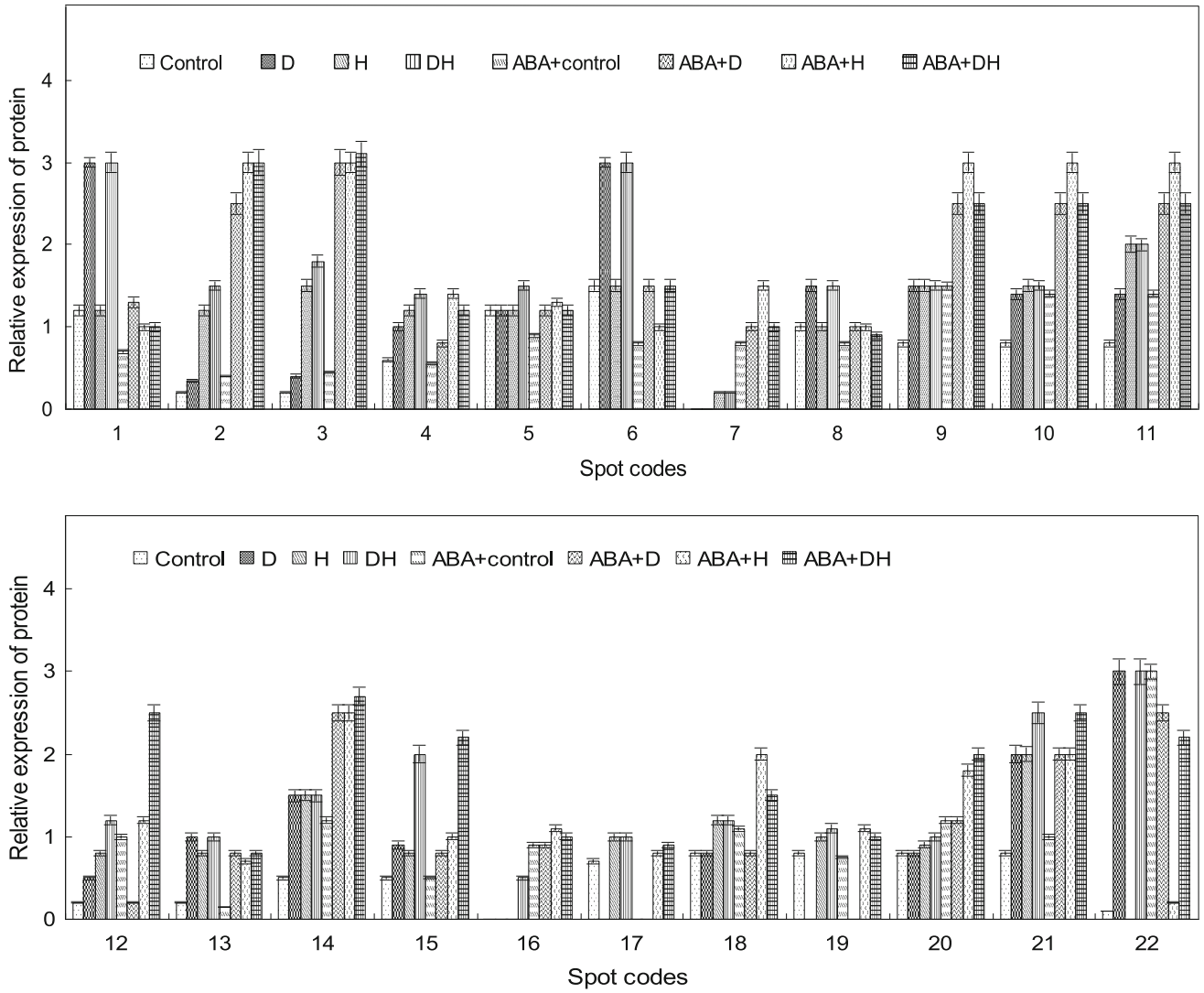

Fig. 2 The *histograms* show the abundance ratio of the identified proteins in response to stress and ABA treatments. Control, distilled water; D, drought; H, heat; DH, drought + heat, ABA + control, 100-μM ABA + distilled water; ABA + D, 100 μM ABA + drought, ABA + H, 100 μM ABA + heat; ABA + DH, 100 μM ABA + drought + heat. The quantitative analysis of the digitized images was performed using the PDQuest software (BioRad, USA) according to the manufacturer's protocols

Compared with the control (Fig. 1a), spots 7, 18 and 20 were up-regulated under heat (Fig. 1c) and combined drought and heat stress (Fig. 1d), but the expression of each protein had no difference among these treatments. Moreover, the up-regulation induced under heat (Fig. 1g) and combined drought and heat stress (Fig. 1h) was enhanced upon pretreatment with 100 μM ABA.

Compared with the control (Fig. 1a), spots 2, 3 9, 10, 11 and 14 were all up-regulated under drought (Fig. 1b), heat (Fig. 1c) and combined heat and drought stress (Fig. 1d). The individual expression of spots 9, 10, 11 and 14 was the same across all of the treatments; however, the increased expression of spots 2 and 3 under the combined stress was the highest, followed by heat stress. Notably, the increased expression under the combined drought and heat stress was nearly equal to the sum of increase under individual treatments of drought and heat stress, which indicated that drought and heat stress had an additive effect on the expression of two proteins under the combination of drought and heat stress. Moreover, the up-regulation induced by drought (Fig. 1f), heat (Fig. 1g) and their combination (Fig. 1h) was enhanced upon the pretreatment with 100 μM ABA.

Compared with the control (Fig. 1a), spots 13, 15 and 21 were all up-regulated by drought (Fig. 1b), heat (Fig. 1c) and their combination (Fig. 1d). The increased expression of spot 13 under drought and combined drought and heat stress had no difference and more than under heat stress alone, respectively. The increased expression of spots 15 and 21 under the combined stresses was far more and less

Table 2 Identification of differentially responsive proteins in maize roots subjected to drought, heat and their combined stresses

Spot	Protein name	Accession no.[a]	Species	Exp. (p*I*/mass)	Theor. (p*I*/mass)	Score[b]	% Cov[c] (matching peptides)
1	Enolase 2 EC = 4.2.1.11	P42895	Maize	5.3/49,000	5.71/48,162	166	45 % (12)
2	Alcohol dehydrogenase 1	P00333	Maize	6.4/40,000	6.28/40,981	93	28 % (7)
3	Alcohol dehydrogenase 1	P00333	Maize	6.7/40,000	6.28/40,981	67	16 % (5)
4	Fructokinase-1	B6U666	Maize	4.8/35,500	4.87/34,603	308	62 % (18)
5	Putative uncharacterized protein	B7ZXQ8	Maize	4.9/34,500	5.35/35,486	109	29 % (7)
6	*O*-Methyltransferase	Q6VWG2	Maize	5.5/38,000	5.48/38,906	164	37 % (10)
7	Putative uncharacterized protein	B4G019	Maize	6.1/34,000	5.96/33,358	132	32 % (7)
8	Caffeoyl-CoA *O*-methyltransferase 1	B4G080	Maize	5.7/28,000	5.09/27,319	135	33 % (7)
9	APx1-cytosolic ascorbate peroxidase	B6TM55	Maize	5.7/245,000	5.56/27,307	249	29 % (12)
10	APx1-cytosolic ascorbate peroxidase	B6UB73	Maize	5.8/24,500	5.65/27,385	179	49 % (10)
11	Putative uncharacterized protein	C0HH17	Maize	6.0/21,000	8.52/40,584	93	24 % (7)
12	Aquaporin PIP2-5	Q9XF58	Maize	6.0/18,000	7.70/29,835	78	14 % (2)
13	Glutathione *S*-transferase 4	P46420	Maize	5.8/23,000	5.77/24,570	137	43 % (12)
14	Putative uncharacterized protein	B4FJF6	Maize	6.0/20,000	9.47/20,052	129	48 % (6)
15	Serine/threonine-protein kinase receptor	B6T9K5	Maize	6.4/19,000	6.80/46,683	49	12 % (4)
16	Pathogenesis-related protein 10	B6SQM6	Maize	5.1/17,000	5.13/17,026	130	54 % (8)
17	VAP27-2	B6TRX1	Maize	5.7/18,000	5.73/43,364	84	23 % (7)
18	DNA polymerase	P10582	Maize	4.8/17,000	8.64/99,999	44	9 % (6)
19	Putative uncharacterized protein	B8A2H7	Maize	5.9/17,000	6.06/23,226	71	17 % (3)
20	Pathogenesis-related protein 10	Q29SB6	Maize	5.0/16,000	5.38/16,942	100	30 % (4)
21	Nucleoside diphosphate kinase	B4FK49	Maize	6.5/15,000	6.30/16,540	73	25 % (5)
22	Glycine-rich RNA-binding protein 2	B6STA5	Maize	6.1/14,000	6.11/15,427	99	64 % (9)

[a] Accession number in the UniProt Knowledgebase (http://www.expasy.org/)

[b] Score is a measure of the statistical significance of a match

[c] Percentage of predicted protein sequence covered by matched peptides

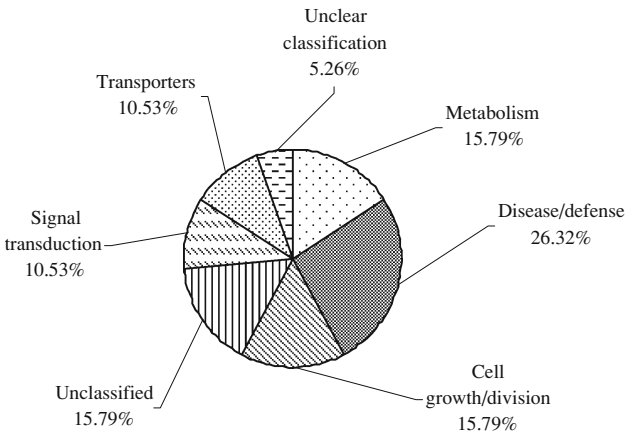

Fig. 3 The functional category distribution of the 22 proteins identified in maize roots subjected to drought, heat and their combination stress

than the sum of their increased expression under drought and heat stress, respectively. In addition, pretreatment with 100 μM ABA did not affect the up-regulation induced by drought (Fig. 1f), heat (Fig. 1g) or their combination (Fig. 1h).

Compared with the control (Fig. 1a), spots 5 and 16 were only up-regulated under the combined drought and heat stress (Fig. 1d). Pretreatment with 100 μM ABA significantly inhibited the up-regulation of spot 5, but enhanced the up-regulation induced by the combined stresses (Fig. 1h).

Compared with the control (Fig. 1a), spots 17 and 19 were inhibited under drought (Fig. 1b) and were induced by heat (Fig. 1c) and the combined stresses (Fig. 1d), although there was no obvious difference in the expression levels. Pretreatment with 100 μM ABA had no effect on the expression of spots 17 and 19 under drought (Fig. 1f), heat (Fig. 1g) or the combined stresses (Fig. 1h).

Compared with the control (Fig. 1a), spot 12 was induced by drought (Fig. 1b), heat (Fig. 1c) and the combined stresses (Fig. 1d), and the level of up-regulation under the combined stress was the highest, followed by the level under heat stress. Notably, the increased expression under the combination of drought and heat stress was

Table 3 The correlation of ABA and stress with the proteins identified in maize roots

	ABA-independent	ABA-inducible	ABA-inhibited
D inducible	Spot no. 22		Spot nos. 1, 6, 8
H inducible		Spot nos. 7, 18, 20[a]	
D, H, DH inducible	Spot nos. 13[a], 15[a], 21[a]	Spots nos. 2[b], 3[b], 9[a], 10[a], 11[a], 14[a]	
DH inducible		Spot no. 16[a]	Spot no. 5[a]
D inhibited	Spot nos. 17[a], 19[a]		

D, H, DH inducible (D, ABA inhibited; DH, H, ABA dependent): spot no. 12

D, H, DH inducible (D, ABA inhibited; DH, H, ABA independent): spot no. 4

D drought, *H* heat, *DH* drought + heat

[a] Drought and heat stress interacted for the induction of these proteins under their combined stresses

[b] Drought and heat stress had an additive effect for the induction of these proteins under their combined stresses

slightly more than the sum of the expression under drought and heat stress. Pretreatment with 100 μM ABA increased the induction by heat (Fig. 1g) and the combination stress (Fig. 1h) but inhibited the induction of protein expression under drought (Fig. 1f).

Compared with the control (Fig. 1a), spot 4 was induced under drought (Fig. 1b), heat (Fig. 1c) and the combined stresses (Fig. 1d), and the level of up-regulation under the combined stress was the highest, followed by the level under heat stress. Notably, the increased expression under the combination of drought and heat stress was less than the sum of that under drought and heat stress treatments individually. Pretreatment with 100 μM ABA did not affect the induction of protein expression by heat (Fig. 1g) but inhibited the induction under drought (Fig. 1f) and the combined stresses (Fig. 1h).

The results of variance analysis showed that, under the combination of drought and heat stress, drought and heat stress had an interactive effect on the protein expression of spot nos. 5, 9, 10, 11, 13–17, 19–21. In summary, it is shown in Table 3 that the effects of ABA and several stress treatments on the expression of the 22 identified proteins, except spots 4 and 12.

Analysis of sHSPs and the effect of ABA on their expression under stress

Previously, we showed that three sHSPs in maize leaves, i.e., sHSP17.2, sHSP17.4 and sHSP26, were up-regulated in maize plants subjected to heat or the combination of drought and heat stress, but not to drought Hu et al. (2010). However, among the 22 proteins identified, no sHSPs were detected in maize roots subjected to the combination of drought and heat stress, potentially as a consequence of their weak expression, which was undetected and therefore not selected for identification using MALDI-TOF MS analysis. Alternatively, there could have been no expression of these sHSPs in the roots upon stress treatments. To investigate the expression of sHSPs in root response to combined drought and heat stress, the expressions of six sHSPs (sHSP16.9, sHSP17.2, sHSP17.4, sHSP17.5, sHSP22 and sHSP26) were investigated at the mRNA level (Fig. 4a). The mRNA expression analysis showed that the six sHSPs were significantly induced under heat stress alone and the combination of drought and heat stress, although there was no difference in their induction effects; however, these sHSPs were faintly expressed under control and drought stress.

To show that the mRNA expressions of the sHSPs were reflected at the protein level, a sHSP26-specific antibody was used to investigate sHSP26 expression in the root response to drought, heat and their combination stress. The results showed that sHSP26 expression were prominently induced under heat and the combination of drought and heat stress, but expression was almost undetectable under the control and drought stress conditions (Fig. 4b), which was similar to the findings for its gene expression. However, because we did not have specific antibodies to the other five sHSPs, we cannot conclude that there are expressions at the protein level for these sHSPs or that it would be similar to their corresponding mRNA expression or to that of sHSP26 when subjected to the same stress.

To examine the effect of ABA on the six sHSPs, pretreatment with 100 μM ABA slightly enhanced the expression of the six sHSPs induced under heat and combined drought and heat stresses at both the mRNA (Fig. 4a) and protein (Fig. 4b) levels; however, the expressions of the six sHSPs were slightly decreased at the mRNA level (Fig. 4a) with little effect at the protein level (Fig. 4b) under drought stress. These results indicated that ABA slightly up-regulated the gene and protein expression of the sHSPs under heat and combined drought and heat stress and slightly down-regulated their expression under drought stress in maize roots.

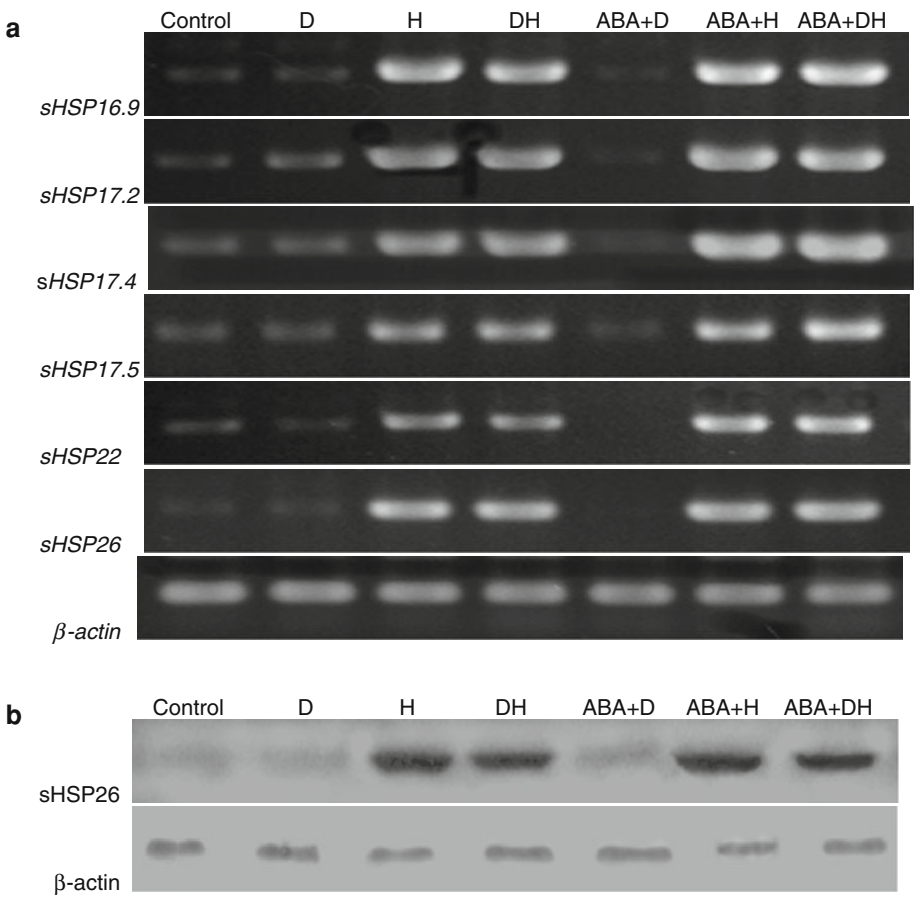

Fig. 4 The analysis of sHSP genes and sHSP26 protein expression in maize roots subjected to drought, heat, and their combined stresses. **a** RNA gel analysis of sHSPs in maize roots subjected to drought, heat and their combined stresses. **b** Western blot analysis of sHSP26 in maize roots subjected to drought, heat and their combined stresses. Subsequent to pretreatment with 100 μM ABA for 5 h, the maize plants were exposed to drought, heat and their combined stresses. Control, distilled water; D, drought; H, heat; DH, drought + heat; ABA + D, 100 μM ABA + drought; ABA + H, 100 μM ABA + heat; ABA + DH, 100 μM ABA + drought + heat. The experiments were repeated at least three times

Discussion

In the field, crops encounter a combination of environmental stresses that might include drought or heat stress. Protein metabolism plays an important role in the plant adaptation to stress, and proteomics offers a powerful approach to identifying the proteins and pathways that are crucial for stress response and tolerance. To the best of our knowledge, there are several studies concerning the proteomics of plant roots in response to drought (Sengupta et al. 2011) or heat stress (Yildiz and Terz-Oglu 2006; Xu and Huang 2008), and a few studies concerning the proteomics of plant roots in response to the combination of drought and heat stress. This study was designed to identify stress proteins induced in roots under these combined stresses. The proteomic response varied among drought, heat and the combination of their stresses in the maize root, and the differentially accumulated proteins have diverse functions, which are discussed below.

Disease/defense

The disease/defense-related proteins were highly abundant. This category contained eight protein spots regulated by the combined stresses, among which spots 2 and 3, 9 and 10, 16 and 20 were the same proteins, respectively. Alcohol dehydrogenases (ADH; EC 1.1.1.1, spots 2 and 3) are a group of proteins that facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NAD^+ to NADH). Maize has two versions of ADH: ADH1 and ADH2. If roots lack oxygen, the expression of *ADH* increases significantly; thus, ADH activity is considered to be essential for the survival of plants during anaerobic conditions (Johnson et al. 1994). ADH expression is also increased in response to drought (Irigoyen et al. 1992), heat stress (Sridhar et al. 2000) and ABA (Hwang and Van-toai 1991). In the present study, ADH1 was significantly up-regulated under the combination of drought and heat stress as a consequence of the interaction of drought and heat stress in maize roots. Moreover, ABA promoted the up-regulation of ADH1 expression under these conditions. Taken together, these results show that ADH1 plays an important role in the root response to environmental stress. To our knowledge, this report is the first to infer a role for ADH1 in plant root response to combined drought and heat stresses.

Spots 9 and 10 were identified as cytosolic ascorbate peroxidase (APX; EC 1.11.1.11), which plays a key role in

regulating H_2O_2 levels and H_2O_2 signaling in plant cells. The up-regulation of APX in the root has also been reported in plants exposed to drought (Selote and Khanna-Chopra 2010), heat (Xu et al. 2010), and their combination (Koussevitzky et al. 2008). In the present investigation, the three stress treatments mediated the up-regulation of APX expression, which was further enhanced upon ABA induction. Interestingly, there was no obvious difference in APX expression among three stress treatments.

The unknown protein (spot 5) has catalytic activity (2 phenolic donors + H_2O_2 = 2 phenoxyl radicals + 2 H_2O) and belongs to the peroxidase family (see http://www.uniprot.org/uniprot/B7ZXQ8). In this study, spot 5 was only stimulated under the combined stresses but was down-regulated upon ABA induction.

Glutathione *S*-transferases (GSTs, EC 2.5.1.18; spot 13) are abundant proteins that are encoded by a highly divergent gene family and have protective functions, such as the detoxification of herbicides and the reduction of organic H_2O_2 formed during oxidative stress. Several studies suggested that GSTs were induced by ABA, drought and heat stress in *Arabidopsis*, maize and barley roots (Kellos et al. 2008; Xu and Huang 2008; Halusková et al. 2009; Jiang et al. 2010). However, other studies showed that GSTs were reduced under drought stress in poplar roots (Plomion et al. 2006). Our present study showed that GST was induced under the three stress treatments in an ABA-independent manner but that drought and heat stress had interactive effect on GST expression under their combined stresses. Increased levels of GST and APX under stress might decrease the production of ROS (such as H_2O_2) and improve root tolerance to stress.

Spots 16 and 20 were identified as pathogenesis-related protein 10 (PR10), which is mainly expressed in root, with low expression in other organs. The increased expression of PR10 has been identified in several proteomics analyses of roots exposed to drought stress (Yang et al. 2011) and exogenous ABA treatment (Colditz et al. 2004). In this study, the abundance of spots 16 and 20 was, respectively, enhanced under heat and the combination of drought and heat stress with up-regulation by ABA induction, suggesting that spots 16 and 20 might be two different PR10-family members. Previous studies also indicated that different PR10 members showed different expression in response to similar stress conditions (Colditz et al. 2004; Xie et al. 2010). Therefore, the expression of different PR10-family members and their regulated profiles suggests that this set of proteins plays a role during root adaptations to various stress conditions.

Increasing data suggest a strong correlation between sHSP accumulation and whole-plant tolerance to stress. In response to heat stress, plants synthesize sHSPs, such as OsHSP26 in rice leaves (Lee et al. 2007) and ZmHSP17.2 in maize (Jorgensen and Nguyen 2004). Our recent study showed that sHSP17.2, sHSP17.4 and sHSP26 were prominently up-regulated under heat and the combination of drought and heat stress in maize leaves but not under drought stress (Hu et al. 2010a). In wheat roots, the proteomics research showed that a number of sHSPs were up-regulated under heat stress and showed different expression profiles with different genotypes or under different temperature treatments (Yildiz and Terz-Oglu 2006). However, in this study, no sHSP was detected using the proteomics approach in maize plants exposed to drought, heat and their combination stresses. The discrepancy potentially resulted from two consequences: (1) the maize roots used in this study were obtained from green seedlings grown under normal light, whereas the previous study (Yildiz and Terz-Oglu 2006) utilized wheat roots from etiolated seedlings under dark conditions; (2) the maize seedlings used in this study were 14 days old, whereas the wheat seedlings in the previous studies were only 2 days old and therefore could have been more sensitive to heat stress than maize, leading to the increased expression of the wheat sHSPs and the subsequent proteomic detection. However, in our study, the results of the RT-PCR showed that the six sHSPs were up-regulated by heat stress and the combination of drought and heat stress. The result of the sHSP26 western blot was similar to that of the six sHSPs under the combination of drought and heat stress. Taken together, these results indicate that sHSPs play a role in plant adaptation to heat stress.

Metabolism

This category included enolase 2 (spot 1), fructokinase (spot 4) and diphosphate kinase (spot 21). Nucleoside diphosphate kinase (NDPKs; EC 2.7.4.6) is one of the key metabolic enzymes that maintain the balance between cellular ATP and other nucleoside triphosphates (NTPs). Recent studies showed that NDPKs played a significant role in the signal transduction pathways of the root response to heat stress (Tang et al. 2008; Xu and Huang 2008). In this study, NDPK was significantly up-regulated by three stress treatments in an ABA-independent manner, whereas there was an interaction between drought and heat stress under their combination stresses.

Enolase 2 (EC 4.2.1.11) is one of the key enzymes that catalyzes the conversion of 2-phosphoglycerate (2-PGA) to PEP during glycolysis. The enzyme enolase was up-regulated in response to drought and ABA treatment (Forsthoefel et al. 1995) but not under heat stress (Van Der Straeten et al. 1991) in maize roots. In this study, the enolase was up-regulated under drought stress but not by ABA treatment.

The enzyme fructokinase (Frk; EC 2.7.1.4) catalyzes the transfer of a high-energy phosphate group to D-fructose to

form fructose-1-phosphate. In roots, it was reported that Frk was down-regulated under NaCl (Sobhanian et al. 2010) and up-regulated under drought and ABA treatments (Bianchi et al. 2002). In this study, Frk was up-regulated under the three stress treatments. Moreover, ABA promoted Frk induction under heat stress and inhibited Frk induction under drought and the combined stresses.

Cell growth/division

In this category, three protein spots were included. *O*-methyltransferase (OMT; spot 6) and caffeoyl-CoA *O*-methyltransferase 1 (COMT; EC 2.1.1.104; spot 8) are involved in lignin biosynthesis (Guillet-Claude et al. 2004; Ma and Xu 2008). Previous results indicated that OMT was not induced under ABA treatment in rice roots (Yang et al. 2004) and was down-regulated under drought stress (Huerta-Ocampo et al. 2011), whereas other studies showed that OMT was highly up-regulated under drought (Yamaguchi et al. 2010). In this study, OMT was up-regulated only under drought stress but was down-regulated by ABA. DNA polymerase (spot 18) is an enzyme that catalyzes the polymerization of deoxyribonucleotides into a DNA strand. The present results showed that DNA polymerase was only up-regulated under heat stress which was further promoted by ABA.

Signal transduction

Serine/threonine-protein kinase receptor (spot 15) belongs to the family of transferases, specifically those that transfer phosphorus-containing groups to protein-serine/threonine kinases. In this study, spot 15 was up-regulated under the three stress treatments, whereas drought and heat stress interacted upon combination. Nevertheless, the induction was not affected by ABA treatment. In addition, this category also included an unknown protein (spot 11), whose function is similar to protein-serine/threonine kinases (http://www.uniprot.org/uniprot/C0HH17). The unknown protein was up-regulated under the three stress treatments and was further induced under ABA treatment. Besides, there was interaction effect between drought and heat stress for the tested protein under the combination of drought and heat stresses.

Transporters

Water channel proteins, aquaporins (PIP), are members of a major (membrane) intrinsic protein (MIP) family and play fundamental roles in transmembrane water movements in plants. Previous studies have shown that heat stress down-regulated the expression of PIP1 and PIP2 (Iglesias-Acostaa et al. 2010), whereas drought stress significantly up-regulated the expression of PIP1-1 and did not affect the expression of PIP2-2 (Vandeleur et al. 2009). Other studies showed that in two *J. curcas* populations, GaoYou CSC63 and YanBian S1, drought stress increased PIP2 expression in GaoYou CSC63, but did not affect PIP2 expression in YanBian S1 (Zhang et al. 2007). In this study, PIP2-5 (spot 12) was up-regulated under the three stress treatments. In addition, the induction under heat and the combination of drought and heat stress was further promoted through ABA treatment, but the expression induced by drought was down-regulated upon ABA treatment. Taken together, these results suggest that PIP family members might play various roles in the plant response to different stresses or respond differently to the same stresses in different plant species.

Spot 17 was identified as a VAP27, which is a SNARE-like protein that might be involved in vesicular transport to or from the ER. In this study, VAP27-2 was inhibited under drought but up-regulated under heat stress with no ABA effect. However, in bean roots, VAP27-2 was not regulated upon drought treatment for 1 and 2 h (Torres et al. 2006).

Unclear classification

This category only contained a glycine-rich RNA-binding protein 2 (spot 22). GRP2 belongs to a superfamily of glycine-rich proteins (GRPs), which are characterized by the presence of semi-repetitive glycine-rich motifs and exhibit developmentally regulated and tissue-specific expression patterns. Previous studies indicated that AtGRP7 was repressed under ABA, high salt and mannitol treatments in *Arabidopsis* roots (Cao et al. 2006). In the present study, GRP2 was up-regulated by drought stress in an ABA-independent manner, and drought stress played a vital role in the regulation of this protein under combined drought and heat stress.

Unclassified

Three unknown proteins (spots 7, 14 and 19) belonged to this category. In this study, spot 7 was up-regulated by heat stress in an ABA-dependent manner; spot 19 was inhibited by drought in an ABA-independent manner; spot 14 was up-regulated by all three stress treatments in an ABA-dependent manner in maize roots.

In summary, root proteomic profiles were detected in maize plants subjected to drought, heat and their combined stresses. More proteins were up-regulated under the combined drought and heat stress than under either stress factor individually. The levels of alcohol dehydrogenase 1, fructokinase-1, aquaporin PIP2-5, serine/threonine-protein kinase receptor, pathogenesis-related protein 10, nucleoside diphosphate kinase and sHSPs were the highest under

the combination of drought and heat stress, as drought and heat stress had interactive or had an additive effect for these proteins. Thus, these proteins could play critical roles in improving root tolerance to drought and heat stresses, and especially their combination stress. The genes encoding these proteins should be further investigated under combined drought and heat stress using molecular approaches, which could provide insights into the molecular basis of root tolerance in maize plants subjected to the combined stresses.

Author contribution Dr. Xiuli Hu designed and supervised the research project and wrote the paper. Dr. Tianxue Liu, Li Zhang, Zuli Yuan, Minghui Lu and Ying Wang contributed in lab experiment, data collection and analysis. Prof. Wei Wang helped in manuscript discussion and editing. All authors have contributed their efforts to this work.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grants No. 30800667 and No. 31171470 to XL Hu), the Fok Ying-Tong Education Foundation, China (Grant No. 122032), the Foundation of Henan Major Public Projects (Grant No. 091100910100) and the State Key Laboratory of Crop Biology (Grant No. 2011KF06).

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