J Plant Growth Regul (2010) 29:455–464
DOI 10.1007/s00344-010-9157-9

Characterization of Small Heat Shock Proteins Associated with Maize Tolerance to Combined Drought and Heat Stress

Xiuli Hu · Yanhui Li · Chaohai Li · Hairong Yang · Wei Wang · Minghui Lu

Received: 13 January 2010 / Accepted: 14 April 2010 / Published online: 22 May 2010

Abstract To investigate how the mechanisms of small heat shock proteins (sHSPs) in regulating maize leaves respond to the combination of drought and heat stress, leaf protein patterns were monitored using a proteomic approach in maize plants exposed to combined drought and heat stress. Two-dimensional electrophoresis (2-DE) was used to identify combined drought- and heat-responsive protein spots in maize leaves. After Coomassie brilliant blue staining, approximately 450 protein spots were reproducibly detected on each gel, wherein 7 protein spots were expressed only under heat and combined drought and heat stress but were almost undetected under control and drought. Using MALDI-TOF mass spectrometry, a total of seven proteins were identified, including cytochrome b6-f complex iron-sulfur subunit, sHSP17.4, sHSP17.2, sHSP26, guanine nucleotide-binding protein β-subunit-like protein, putative uncharacterized protein, and granule-bound starch synthase IIa. Moreover, the gene expression of three sHSPs was analyzed at the transcriptional level and indicated that all three sHSPs were expressed under several treatments although their expression levels were obviously more enhanced by heat and combined drought and heat stress than by control and drought. In investigations of the effect of abscisic acid (ABA) on the three sHSPs, pretreatment with 100 μM ABA enhanced substantially the expression of the three sHSPs at the protein level, but only slightly at the mRNA level. These results show that transcription levels are not completely concomitant with translation and suggest that ABA induces the post-transcriptional regulation of sHSP17.2, sHSP17.4, and sHSP26 expression, which can lead to a better understanding of the mechanisms of plant response to the combination of drought and heat stress.

Keywords ABA · Combined drought and heat stress · sHSP17.2 · sHSP17.4 · sHSP26 · *Zea mays* L

Abbreviations

ABA	Abscisic acid
CBB	Coomassie brilliant blue
cAPX	Cytosolic ascorbate peroxidase
DTT	Dithiothreitol
IEF	Isoelectric focusing
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MS	Mass spectrometry
PMSF	Phenylmethanesulfonyl fluoride
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
pI	Isoelectric point
sHSPs	Small heat shock protein
T	Tungstate
2-DE	Two-dimensional electrophoresis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid

X. Hu (✉) · Y. Li · H. Yang · W. Wang · M. Lu
College of Life Science, Henan Agricultural University, Zhengzhou 450002, China
e-mail: xiulihu@126.com

C. Li
College of Agronomy, Henan Agricultural University, Zhengzhou 450002, China

C. Li
Huanghuaihai Regional Innovation Center for Maize Technology, Ministry of Agriculture, Zhengzhou, China

Introduction

Abiotic stresses usually lead to protein dysfunction. It is especially important for plant survival under stress to maintain proteins in their functional conformations and prevent the aggregation of non-native proteins. Heat shock proteins (HSPs) as chaperones are responsible for protein folding, assembly, translocation, and degradation in many normal cellular processes, stabilize proteins and membranes, and can assist in protein refolding under stress conditions (Low and others 2000; Yu and others 2005; Jaya and others 2009). They can play a crucial role in protecting plants against stress by reestablishing normal protein conformation and thus cellular homeostasis (for review see Wang and others 2004).

The major HSPs synthesized by eukaryotes, including plants, are divided into five conserved classes based on their molecular weight: HSP100, HSP90, HSP70, HSP60, and small heat shock proteins (sHSPs). sHSPs range in size from 15 to 42 kDa and are characterized by a conserved sequence at their C terminus (Sun and others 2002). The α-crystallin-related sHSPs are ubiquitous in nature, but 20–40-kDa sHSPs are unusually abundant in higher plants. sHSPs are divided into six classes based on their sequence alignments, immunological cross reactivity, and cellular compartmentalization. Three classes (I, II, and III) are localized in the cytoplasm or the nucleus and the other three are localized in the chloroplast, the endoplasmic reticulum, and the mitochondria (Sun and others 2002; Vierling 1991).

Although sHSPs' precise functional mechanism is still unclear, in vivo studies have shown that sHSPs act as molecular chaperones (Low and others 2000; Yu and others 2005; Jaya and others 2009). Plants synthesize significant amounts of sHSPs when exposed to high temperatures (Ahn and Zimmerman 2006; Charng and others 2006; Volkov and others 2006), drought stress (Sato and Yokoya 2008; Zahur and others 2009), oxidative stress (Neta-Sharir and others 2005; Volkov and others 2006), cold acclimation (Guo and others 2007), salts (Sun and others 2001), and ABA treatment (Yu and others 2005; Zahur and others 2009; Zou and others 2009). These results suggest that sHSPs play an important part in plant endurance to abiotic stress. However, to the best of our knowledge, relatively little is known about the physiological function of sHSPs under the combination of drought and heat stress. Environmental stresses such as salt, drought, cold, and extreme temperatures severely limit crop productivity. In particular, global climate change in the form of rising temperatures and altered soil moisture may result in a more than 50% loss of food crops by the year 2050 (Bray and others 2000; Thomson and others 2005). Because each sHSP gene has a unique sequence with the potential for a special function, the unusual abundance of sHSPs (20–40 kDa) in plants could reflect their functional diversity. This further suggests possible functional differences among plant sHSPs in response to the combination of drought and heat stress. Therefore, knowledge of sHSPs' response to the combination of drought and heat stress is crucial for us to understand the mechanism of crop acclimation to abiotic stress in nature.

Responses to environmental stresses in higher plants are controlled by a complex signal cascade of ABA-dependent and -independent signaling pathways. ABA accumulation in plants during stress is involved in regulatory pathways mediating defense responses (Hu and others 2008, 2010). Enhanced sHSP expression has been reported in plant responses to ABA treatment (Sato and Yokoya 2008; Yi and others 2006; Zou and others 2009). However, the molecular mechanism of the interaction of sHSPs and ABA in response to combined drought and heat stress is not fully understood.

Proteomics is a powerful tool for separating complex protein mixtures and has been used to analyze protein changes in plant responses to abiotic stresses. The aim of this study was to identify maize leaf sHSPs that are associated with combined drought and heat stress using a comparative proteomic approach and to further confirm the correlations between ABA and sHSPs in maize seedlings exposed to combined drought and heat stress.

Materials and Methods

Plant Material and Stress Treatments

Seeds of maize (*Zea mays* L., cv. Zhengdan 958) were used in the experiments. Currently, Zhengdan 958 is one of the high-yield maize hybrids in China. Seeds were washed in distilled water and germinated on moistened filter papers after seeds were surface-sterilized for 10 min in 2% hypochlorite. Maize seedlings were grown in Hogland's nutrient solution at 400 μmol m^{-2} s^{-1} photosynthetically active radiation, a 14/10-h day/night cycle, a day/night temperature of 28/22°C, and a relative humidity of 75% in a light chamber. When the second leaves were fully expanded, seedlings were subjected to various treatments.

Drought stress was imposed by placing the seedlings in a PEG solution (–0.7 MPa) for 8 h at 28°C and 40% relative humidity. Heat shock was applied by raising the temperature from 28 to 42°C at an interval 2°C/h and then kept at 42°C for 1 h, for a total of 8 h. Each stress treatment was therefore 8 h. The combination stress consisted of the imposition of PEG treatment and heat shock simultaneously. Control seedlings were kept at 28°C and 75% relative humidity. Afterward, expanding leaves (the

second from the top) of treated and untreated seedlings were sampled, frozen immediately in liquid N_2, and stored at –80°C until analysis.

In addition, for inhibitor and inducer experiments, maize seedlings were pretreated with 2 mM tungstate (T) and 100 μM ABA, respectively, for 5 h and then exposed to stress treatments for 8 h and sampled as described above.

Maize Leaf Protein Extraction

Maize leaf samples were homogenized in liquid N_2 in a mortar, and soluble protein was extracted by SDS/phenol extraction protocol as described in Wang and others (2006).

Two-dimensional Electrophoresis

Proteins were analyzed by two-dimensional gel electrophoresis as described by Wang and others (2006). Isoelectrofocusing was performed in an Ettan III system (GE Healthcare, Piscataway, NJ, USA) using pH 4-7 IPG strips (7 cm; GE Healthcare). The second dimension was carried out in 12.5% SDS polyacrylamide gels. 2-DE gels were stained with colloidal CBB G. Digital images of the gels were obtained by using an ImageScanner.

Protein Identification by Mass Spectrometry

Proteins of interest in stained 2-DE gels were subjected to in-gel digestion with trypsin (Wang and others 2009). The digested fragments were analyzed on an Ettan MALDI-TOF Pro mass spectrometer (GE Healthcare). The ion acceleration voltage was 20 kV. Each spectrum was internally calibrated with the masses of two trypsin autolysis products. Mass spectra were used to search the UniProt Knowledgebase (Swiss-Prot and TrEMBL, http://www.expasy.org/) for homologous sequences with Mascot software (http://www.matrixscience.com). Theoretical Mr and pI of identified proteins were predicted at http://www.expasy.ch/tools/pI_tools.html.

Gene Expression Analysis by RT-PCR

Total RNA was isolated from leaves by using RNeasy mini kit according to the instructions supplied by the manufacturer (Qiagen, Valencia, CA). Approximately 3 μg of total RNA was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was amplified by PCR using the following primers: sHSP17.2, forward 5′-GTTCTGTTTGTTGAGACGCATAG-3′ and reverse 5′-ACCCGCAAGATTGAGATTGT-3′; sHSP17.4, forward 5′-AAAACTCGACCAACAATGTCGCT-3′ and reverse 5′-ACACTGATACACGACGGATGAGA-3′; sHSP26, forward 5′-CAGATGCTGGACACGATGGA-3′ and reverse 5′-CCTTGCTCTTGTCGCACTCAT-3′; and cytosolic ascorbate peroxidase (*cAPX*), forward 5′-TCGGCACCATGAAGAACCC and reverse TCCTCGTCCGCTGCGTATT-3′. To standardize the results, the relative abundance of β-actin was also determined and used as the internal standard. Aliquots of the PCR reactions were loaded on agarose gels and stained with ethidium bromide.

Results

2-DE Analysis of Maize Leaf Proteins

To investigate the unique expressed protein of the maize leaf response to the combination of drought and heat stress in comparison with drought stress and heat stress alone, proteins were extracted from the second leaf and separated by 2-DE after 2-week-old maize seedlings were subjected to drought, heat and combined drought and heat stress for 8 h. A 2-DE gel pattern, in a pI range of 4-7, was detected by CBB staining. More than 450 protein spots were reproducibly detected in each CBB-stained gel by 2-DE analysis of protein samples (Fig. 1). Among these proteins, no specific protein was detected in plants subjected to the combination of drought and heat stress as expected, but seven proteins (spots 1, 2, 3, 4, 5, 6, and 7) were detected only in plants subjected to heat stress (Fig. 1A, c) and combined drought and heat stress (Fig. 1A, d), but were almost undetected under control (Fig. 1A, a) and drought (Fig. 1A, b).

Identification of Heat-responsive Protein Spots

To identify the differentially expressed proteins, spots were excised from the preparative gels, in-gel digested by trypsin, and analyzed using a MALDI-TOF MS. The seven proteins resolved from the samples were identified. The database search results are given in Table 1. All seven spots contained only one protein. The spot 1-7 proteins were respectively identified as a cytochrome b6-f complex iron-sulfur subunit (EC 1.10.99.1) protein (UniProt accession No. B4F9N4), a guanine nucleotide-binding protein β-subunit-like protein (B6SJ21), a putative uncharacterized protein (B4G072), a granule-bound starch synthase IIa (A4URH2), and three sHSPs: sHSP17.4 (B6TDB5), sHSP17.2 (Q43701), and sHSP26 (Q41815). The identified proteins were classified into the specific groups according to putative physiological functions: (1) sHSPs, (2) energy- and metabolism-related proteins, and (3) regulatory proteins. sHSPs (42.8%) and energy metabolism (42.8%) were the largest functional category, suggesting that energy and metabolic pathways are highly disrupted and sHSPs may

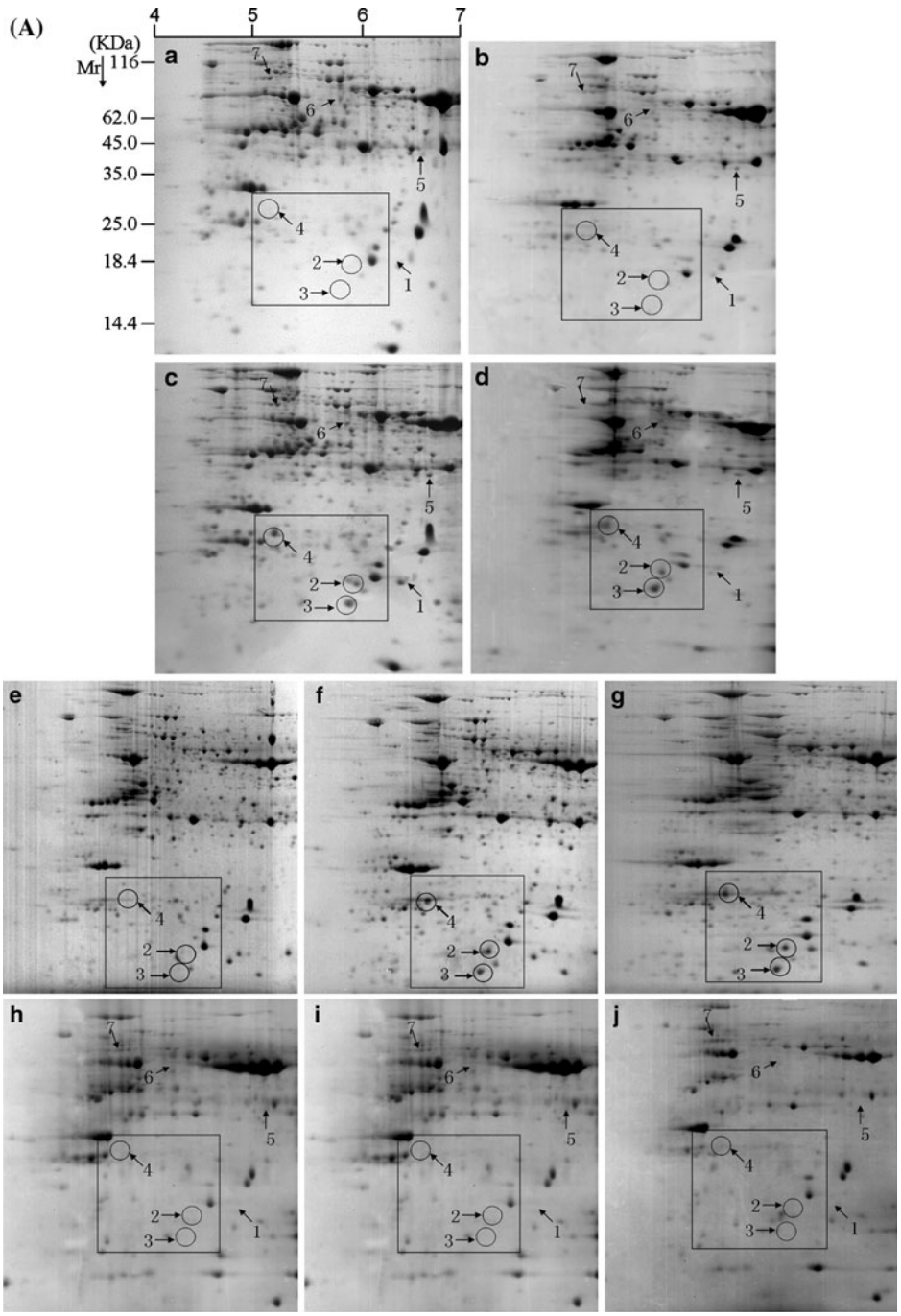

Fig. 1 A 2-DE analysis of maize leaf proteins. **A** 2-DE gel of maize leaf proteins exposed to drought, heat, and combined drought and heat stress and the effect of ABA on maize leaf proteins exposed to several treatments. After pretreatment with 100 μM ABA or ABA inhibitor 2 mM T for 5 h, the maize plants were exposed to drought, heat, and the combination of drought and heat. Protein loads were 800 μg. Gels were CBB G stained. Seven leaf proteins were subjected to MALDI-TOF analysis. Three sHSPs located in the circled areas (spot 2-4) and another four proteins indicated by arrows were expressed only in response to heat and the combination of drought and heat stress. (a) Control (distilled water); (b) drought; (c) heat; (d) combined drought and heat stress; (e) 100 μM ABA + drought; (f) 100 μM ABA + heat; (g) 100 μM ABA + drought + heat; (h) 2 mM T + drought; (i) 2 mM T + heat; (j) 2 mM T + drought + heat. **B** Magnified views of square frame region of 2-DE gels, highlighting the different expressions of the sHSP17.4 (spot 2, circled), sHSP17.2 (spot 3, circled), and sHSP26 (spot 4, circled). This is a representative figure from three biological replicas. **C** Histograms show the abundance ratio of the identified sHSP17.2, sHSP17.4, and sHSP26 in (**A**). CK, distilled water (control); 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; T + D, 2 mM T + drought; T + H, 2 mM T + heat; T + DH, 2 mM T + drought + heat. Each value represents the average of duplicate 2D gels

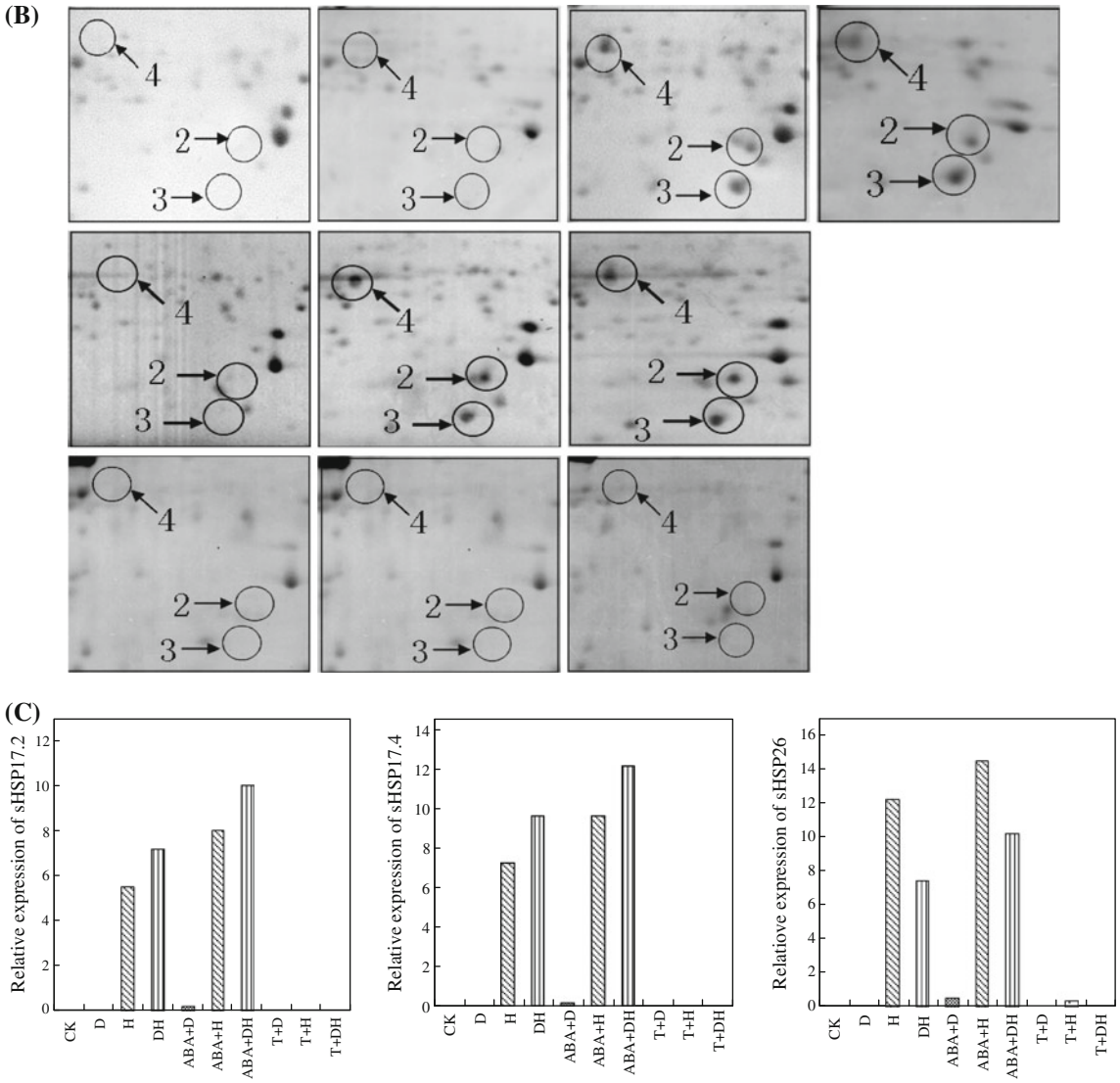

Fig. 1 continued

Table 1 Identification of differentially responsive proteins in maize leaves subjected to drought, heat, and combination of drought and heat stress

Spot	Protein name	Accession No.	Exp. pI/mass	Theor. pI/mass	Score[b]	%cov[c] (matching peptides)
1	Cytochrome b6-f complex iron-sulfur subunit (EC 1.10.99.1)	B4F9N4[a]	6.5/20000	8.58/24324	466	34.1(5)
2	Hsp17.4	B6TDB5[a]	5.8/20000	5.55/17775	269	18.8(2)
3	Hsp17.2	Q43701[a]	5.7/17500	5.54/17152	304	45.4(4)
4	Hsp26	Q41815[a]	5.2/27000	7.86/26361	479	35.4(6)
5	Guanine nucleotide-binding protein β-subunit-like protein	B6SJ21[a]	6.5/40000	6.13/36670	694	12.0(3)
6	Putative uncharacterized protein	B4G072[a]	5.8/70000	5.15/50328	922	14.3(4)
7	Granule-bound starch synthase IIa	A4URH2[a]	5.2/90000	5.81/66914	560	21(2)

[a] Accession number in 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

play an important role in protecting the cells from damage under heat and combined drought and heat stress.

Analysis of sHSPs at mRNA Level

Among all identified proteins, three sHSPs, that is, sHSP17.2, sHSP17.4, and sHSP26, were selected for investigation of their expression patterns in response to drought, heat, and combined drought and heat stress at the mRNA level. To better evaluate their mRNA expression levels, we first ran PCR reactions with increasing numbers of cycles and quantitatively analyzed the sHSP bands on the RNA gel by the UVISoft UVIBand software (Fig. 2A). Based on Fig. 2A, the optimal number of cycles of PCR reactions for sHSP17.2, sHSP17.4, and sHSP26 was 28, 28, and 30, respectively. The RNA gels of sHSP17.2, sHSP17.4, and sHSP26 are shown in Fig. 2B. On the other hand, Fig. 2A and B also show that sHSP17.2, sHSP17.4, and sHSP26 were all expressed at mRNA levels under control, drought, heat, and the combination of drought and heat stress, although the expression levels of the three sHSPs were enhanced by heat stress and combined drought and heat stress more than by control and drought. However, at the proteomic level, sHSP17.2, sHSP17.4, and sHSP26 were expressed only in response to heat and combined drought and heat stress (Fig. 1). Taken together, these data indicate that the mRNA levels of the three sHSPs showed different expression patterns from those of the corresponding proteins.

The Effect of ABA on the Expression of sHSPs

The ABA biosynthetic inhibitor T, which was shown to block the formation of ABA from abscisic aldehyde by impairing abscisic aldehyde oxidase (Hansen and Grossmann 2000), was used to determine the effects of ABA on the three sHSPs in the leaves of maize plants exposed to drought, heat, and the combination of drought and heat stress. Pretreatment with 2 mM T blocked substantially the expression of sHSP17.2, sHSP17.4, and sHSP26 in maize leaves induced by heat and the combination of drought and heat stress at the protein level (Fig. 1A, h-j), but only slightly at the mRNA level (Fig. 2). However, since the T treatment could have inactivated other important Mo-containing enzymes (for example, nitrate reductase) and signaling components, a second strategy was employed to further evaluate the involvement of ABA as a regulator of the three sHSPs in maize leaves. This alternative approach consisted of supplementing the growth medium of the plants with 100 μM ABA. In contrast to T treatments, exogenous ABA significantly enhanced the expression of sHSP17.2, sHSP17.4, and sHSP26 in maize leaves induced by heat and the combination of drought and heat stress at the protein level (Fig. 1A, e-g), but only slightly at the mRNA level (Fig. 2). Besides, the abundance ratio of the three sHSPs in the 2-DE gel was obviously related to ABA (Fig. 1C), but only slightly related to ABA in the RNA gel (Fig. 2C).

Research shows that cAPX can be dominantly induced by drought (Hu and others 2008), heat, and the combination of drought and heat stress (Gong and others 1998; Rizhsky and others 2002), and ABA may increase drought and heat tolerance in maize seedling by maintaining higher activities of cAPX and the expression of the *cAPX* gene (Gong and others 1998; Hu and others 2008). Thus, the *cAPX* gene was used as a positive control to further demonstrate the effectiveness of ABA, inhibitor, drought, or heat treatments. The results showed that pretreatment with 100 μM ABA obviously enhanced the expression of the *cAPX* gene of maize leaves induced by drought, heat, and combined stress. In contrast, pretreatment with 2 mM T significantly blocked the expression of the *cAPX* gene induced by drought, heat, and combined stress in maize leaves (Fig. 2B, d). This validated the data about the effect of ABA on sHSP17.2, sHSP17.4, and sHSP26.

Therefore, the data obtained strongly suggest that ABA might be involved only in the post-transcriptional regulation of sHSP17.2, sHSP17.4, and sHSP26 expression in maize leaves exposed to heat and the combination of drought and heat stress and has no influence on their gene expression.

Discussion

Plants are frequently subject to a wide array of adverse stresses and, unlike animals, are unable to move away. Therefore, plants have evolved mechanisms to overcome different types of stress, including heat stress and drought stress. sHSPs are the most abundant HSPs and unique in higher plants, because other eukaryotes have far fewer sHSPs (Vierling 1991). The high diversification of plant sHSPs probably reflects a molecular adaptation to a wide range of environmental stresses, including heat, cold, drought, salinity, and oxidative stress (for review see Wang and others 2004).

Increasing data suggest a strong correlation between sHSP accumulation and whole-plant tolerance to stress. Plants obviously synthesize sHSPs in response to heat stress, and it was indicated that heat stress strongly induces the expression of *Arabidopsis* Hsa32 (Charng and others 2006), AtHSP17.6A (Papdi and others 2008), AtHSP25.3, AtHSP18.2, and HSP40 (Yang and others 2009), rice OsHSP26 (Lee and others 2000), maize ZmHSP17.2 (Jorgensen and Nguyen 2004), tomato LeHSP17.4-CII (Frank and others 2009), tomato GHSP26 (Zahur and others 2009), HSP26.6 (Lee and others 1998), and HSP21

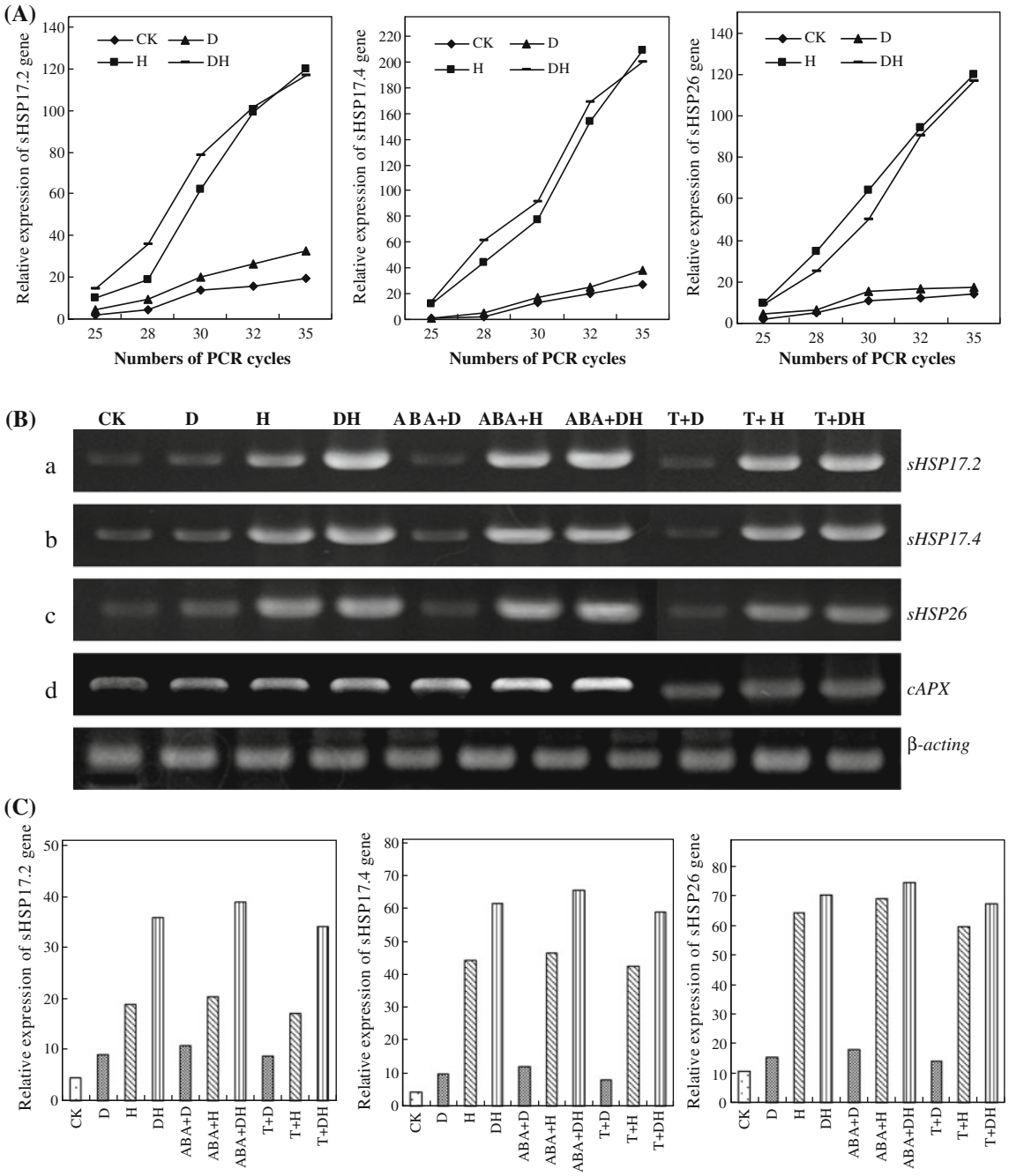

Fig. 2 RNA gel analysis of sHSP17.2, sHSP17.4, and sHSP26 of maize leaves in response to drought, heat, and combination of drought and heat stress and the effect of ABA on the expression of three sHSPs genes. **A** The relative levels of sHSP17.2, sHSP17.4, and sHSP26 genes in RNA gel determined by PCR reactions with increasing number of cycles are shown. **B** The effect of ABA on the gene expression of sHSP17.2, sHSP17.4, and sHSP26 of maize leaves exposed to drought, heat, and combination of drought and heat stress. **C** Histograms show the relative levels of sHSP17.2, sHSP17.4, and sHSP26 genes in (**B**). After pretreatment with 100 μM ABA or ABA inhibitor 2 mM T for 5 h, the maize plants were exposed to drought, heat, and the combination of drought and heat. CK, distilled water (control); 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; T + D, 2 mM T + drought; T + H, 2 mM T + heat; T + DH, 2 mM T + drought + heat. Experiments were repeated at least three times

(Osteryoung and Vierling 1994). Studies using transgenic plants have shown that enhanced heat tolerance in higher plants is correlated with carrot *HSP17.7* constitutive expression in transgenic potato (Ahn and Zimmerman 2006), overproduction of *HSP17.7* in rice (Sato and Yokoya 2008), and tomato *MT-sHSP* overexpression into tobacco (Sanmiya and others 2004). These results suggest that sHSPs play an important part in thermotolerance.

Some data indicate that the enhanced drought tolerance in higher plants is correlated with the accumulation of sHSPs such as *Phaseolus vulgaris* PvHSP17-19 (Colmenero-Flores and others 1997), spruce HSP23.5 and HSPl7.1 (Dong and Dunstan 1996), tobacco GHSP26 (Zahur and others 2009), cotton GHSP26 (Maqbool and others 2007), sunflower *HSP17.6* (Almoguera and Jordano 1994), and overproduction of sHSP17.7 in rice (Sato and Yokoya 2008).

These results concentrated mostly on the relationships between sHSPs and plant adaptation to drought or heat single-stress factors. However, the data have shown that the response of *Arabidopsis* plants to a combination of drought and heat stress was distinct from that of plants subjected to drought or heat stress. Transcriptome analysis of *Arabidopsis* plants subjected to a combination of drought and heat stress revealed a new pattern of defense response in plants: The transcription of 14 different *sHSP*s was enhanced under combined drought and heat stress, 13 under heat, and 2 under drought. Except for *sHSP40* expression under combined stress, the other 13 *sHSP*s (17-23.6) were completely in agreement with the 13 *sHSP*s under heat; enhanced transcription of *sHSP17* and *sHSP20* under drought was found under heat and the combination of drought and heat stress (Rizhsky and others 2004). But more needs to be known about the mechanism of action of sHSPs in enhancing plant endurance to the combination of drought and heat stress.

Although several proteomic analyses have been carried out to analyze maize leaf proteome expression under drought or heat stress, to the best of our knowledge there have not been any reports on the combination of drought and heat stress. In the present study, sHSP17.4, sHSP17.2, and sHSP26 were found only under heat stress and the combination of heat and drought stress by using a proteomics approach but were almost undetected under control and drought stress. Three sHSPs were all expressed at mRNA levels under several treatments although their expression levels were obviously enhanced by heat and combined drought and heat stress more than by control and drought. In addition, our results showed that heat stress-increased sHSPs were all found under the combination of drought and heat stress, which is agreement with the results of Rizhsky and others (2004). However, the three sHSPs identified by proteomic analyses in the present study were not found in the results obtained by Rizhsky and others (2004) by transcriptome profiling. The discrepancy is possibly a result of (1) the difference between the response of maize and *Arabidopsis* to stress, and (2) the transcription patterns not being directly concomitant with protein expression levels, which is supported by our present study and the previously established concept (Agrawal and Rakwal 2006; Lee and others 2007). Therefore, it might be more important to analyze the plant response to combined drought and heat stress from proteomic analyses.

Exogenous ABA treatment can also induce the expression of *Phaseolus vulgaris PvHSP17-19* (Colmenero-Flores and others 1997), spruce *HSP23.5* and *HSPl7.1* (Dong and Dunstan 1996), *Arabidopsis AtHSP17.6A* (Papdi and others 2008), tobacco *GHSP26* (Zahur and others 2009), rice *HSP71.1* (Zou and others 2009), and sunflower *HSP17.6* (Almoguera and Jordano 1994). Our present study showed that ABA had significant influence on the expression of three sHSPs at the protein level, but only slightly at the mRNA level. These results show that transcription levels are not completely concomitant with translation and provide new insights that ABA induces post-transcriptional regulation of *sHSP17.2*, *sHSP17.4*, and *sHSP26* expression, which can lead to a better understanding of the molecular basis of the plant response to the combination of drought and heat stress.

We analyzed subcellular localization of sHSP17.2, sHSP17.4, and sHSP26 using MultiLoc (http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc/) and WoLF PSORT (http://wolfpsort.org/), and supposed that the target location of sHSP17.2 and sHSP17.4 might be the cytoplasm or the nucleus, whereas chloroplasts might be the target location of sHSP26. Recent studies indicate that chloroplast sHSPs (21-30 kDa) play an important role in heat tolerance (for review see Huang and Xu 2008). But what roles sHSP26 plays in protection of PSII and PSI during the combination of drought and heat stress remains to be investigated.

The results of this study suggest that plants cope with combined drought and heat stress in a complex manner, where sHSPs play a pivotal role in this complex cellular network. Although this study is an initial proteomic investigation into the maize leaf response to the combination of drought and heat stress, it is our belief that this kind of study provides a good starting point in understanding plant responses to combined stress. However, further analyses should be conducted to gain a better understanding of the overall responses of plants to combined stress.

Acknowledgments This work was supported by the National Natural Science Foundation of China (grant No. 30800667 to X. L. Hu), the China Postdoctoral Science Foundation (grant Nos. 20080440824 and 200902357 to X. L. Hu), the Natural Science Foundation of Henan Educational Committee (grant No. 2008A180011 to X. L. Hu), the Foundation for University Key Teacher by the Ministry of Education (grant No. 2009GGJS-028 to X. L. Hu), and the Foundation of Henan Major Public Projects (grant No. 091100910100).

References

Agrawal GK, Rakwal R (2006) Rice proteomics: a cornerstone for cereal food crop proteomes. Mass Spectrom Rev 25:1–53

Ahn YJ, Zimmerman JL (2006) Introduction of the carrot HSP17.7 into potato (*Solanum tuberosum* L.) enhances cellular membrane stability and tuberization *in vitro*. Plant Cell Environ 29:95–104

Almoguera C, Jordano J (1994) Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs. Biotechnol Appl Biochem 19:41–49

Bray EA, Bailey-Serres J, Weretilnyk E (2000) Responses to abiotic stresses. In: Gruissem W, Buchannan B, Jones R (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, MD, pp 1158–1249

Charng YY, Liu HC, Liu NY, Hsu FC, Ko SS (2006) Arabidopsis Hsa32, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. Plant Physiol 140:1297–1305

Colmenero-Flores JM, Campos F, Garciarrubio A, Covarrubias AA (1997) Characterization of *Phaseolus vulgaris* cDNA clones responsive to water deficit: identification of a novel late embryogenesis abundant-like protein. Plant Mol Biol 35:393–405

Dong JZ, Dunstan DI (1996) Characterization of three heat-shock-protein genes and their developmental regulation during somatic embryogenesis in white spruce [*Picea glauca* (Moench) Voss]. Planta 200:85–91

Frank G, Pressman E, Ophir R, Althan L, Shaked R, Freedman M, Shen S, Firon N (2009) Transcriptional profiling of maturing tomato (*Solanum lycopersicum* L.) microspores reveals the involvement of heat shock proteins, ROS scavengers, hormones, and sugars in the heat stress response. J Exp Bot 60: 3891–3908

Gong M, Li YJ, Chen SZ (1998) Abscisic acid induced thermotolerance in maize seedlings is mediated by Ca^{2+} and associated with antioxidant systems. J Plant Physiol 153:488–496

Guo SJ, Zhou HY, Zhang XS, Li XG, Meng QW (2007) Overexpression of CaHSP26 in transgenic tobacco alleviates photoinhibition of PSII and PSI during chilling stress under low irradiance. J Plant Physiol 164:126–136

Hansen H, Grossmann K (2000) Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol 124:1437–1448

Hu XL, Jiang MY, Zhang JH, Tan MP, Zhang AY (2008) Cross-talk between Ca^{2+}/CaM and H_2O_2 in abscisic acid-induced antioxidant defense in leaves of maize plants exposed to water stress. Plant Growth Regul 55:183–198

Hu XL, Liu RX, Li YH, Wang W, Tai FJ, Li CH (2010) Heat shock protein 70 regulates the abscisic acid–induced antioxidant response of maize to combined drought and heat stress. Plant Growth Regul 60(3):225–235

Huang B, Xu CP (2008) Identification and characterization of proteins associated with plant tolerance to heat stress. J Integr Plant Biol 50:1230–1237

Jaya N, Garcia V, Vierling E (2009) Substrate binding site flexibility of the small heat shock protein molecular chaperones. Proc Natl Acad Sci USA 106:15604–15609

Jorgensen JA, Nguyen HT (2004) Isolation, sequence and expression of a cDNA encoding a Class I heat shock protein (HSP17.2) in maize. Plant Sci 97:169–175

Lee BH, Tanaka Y, Iwasaki T, Yamamoto N, Kayano T, Miyao M (1998) Evolutionary origin of two genes for chloroplast small heat shock protein of tobacco. Plant Mol Biol 37:1035–1043

Lee BH, Won SH, Lee HS, Miyao M, Chung Kim WJ, Jo J (2000) Expression of the chloroplast-localized small heat shock protein by oxidative stress in rice. Gene 245:283–290

Lee DG, Ahsan N, Lee SH, Kang KY, Bahk JD, Lee IJ, Lee BH (2007) A proteomic approach in analyzing heat-responsive proteins in rice leaves. Proteomics 7:3369–3383

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

Maqbool A, Zahur M, Irfan M, Qaiser U, Rashid B, Husnain T, Riazuddin S (2007) Identification, characterization and expression of drought related alpha-crystalline heat shock protein gene (*GHSP26*) from Desi Cotton. Crop Sci 47:2437–2444

Neta-Sharir I, Tal Isaacson T, Lurie S, Weissa D (2005) Dual role for tomato heat shock protein 21: protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. Plant Cell 17:1829–1838

Osteryoung KW, Vierling E (1994) Dynamics of small heat shock protein distribution within the chloroplasts of higher plants. J Biol Chem 269:28676–28682

Papdi C, Ábrahám E, Joseph PM, Cristina Popescu C, Koncz C, Szabados L (2008) Functional identification of *Arabidopsis* stress regulatory genes using the controlled cDNA overexpression system. Plant Physiol 147:528–542

Rizhsky L, Liang H, Mittler R (2002) The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol 130:1143–1151

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S (2004) When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. Plant Physiol 134:1683–1696

Sanmiya K, Suzuki K, Egawa Y, Shono M (2004) Mitochondrial small heat-shock protein enhances thermotolerance in tobacco plants. FEBS Lett 557:265–268

Sato Y, Yokoya S (2008) Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17.7. Plant Cell Rep 27:329–334

Sun W, Bernard C, van de Cotte B, Montagu MV, Verbruggen N (2001) *At-HSP17.6A*, encoding a small heat-shock protein in *Arabidopsis*, can enhance osmotolerance upon overexpression. Plant J 27:407–415

Sun W, Van Montagu M, Verbruggen N (2002) Small heat shock proteins and stress tolerance in plants. Biochim Biophys Acta 1577:1–9

Thomson AM, Brown RA, Rosenberg NJ, Izaurralde RC, Benson V (2005) Climate change impacts for the conterminous USA: an integrated assessment. Part 3. Dryland production of grain and forage crops. Clim Change 69:43–65

Vierling E (1991) The role of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42:579–620

Volkov RA, Panchuk II, Mullineaux PM, Schöffl F (2006) Heat stress-induced H_2O_2 is required for effective expression of heat shock genes in *Arabidopsis*. Plant Mol Biol 61:733–746

Wang WX, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 9:244–252

Wang W, Vignani R, Scali M, Cresti M (2006) A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Electrophoresis 27:2782–2786

Wang W, Bianchi L, Scali M, Liu LW, Bini L, Cresti M (2009) Proteomic analysis of β-1, 3-glucanase in grape berry tissues. Acta Physiol Plant 31:597–604

Yang KZ, Xia C, Liu XL, Dou XY, Wang W, Chen LQ, Zhang XQ, Xie LF, He L, Ma X, Ye D (2009) A mutation in Thermosensitive Male Sterile 1, encoding a heat shock protein with DnaJ and PDI domains, leads to thermosensitive gametophytic male sterility in *Arabidopsis*. Plant J 57:870–882

Yi SY, Sun AQ, Sun Y, Yang JY, Chun-Mei Zhao, Liu J (2006) Differential regulation of Lehsp23.8 in tomato plants: analysis of a multiple stress-inducible promoter. Plant Sci 171:398–407

Yu JH, Kim KP, Park SM, Hong CB (2005) Biochemical analysis of a cytosolic small heat shock protein, NtHSP18.3, from *Nicotiana tabacum*. Mol Cells 19:328–333

Zou J, Liu A, Chen X, Zhou X, Gao G, Wang W, Zhang X (2009) Expression analysis of nine rice heat shock protein genes under abiotic stresses and ABA treatment. J Plant Physiol 166:851–861

Zahur M, Maqbool A, Irfan M, Khan BMY, Qaiser U, Rashid B, Husnain T, Riazuddin S (2009) Functional analysis of cotton small heat shock protein promoter region in response to abiotic stresses in tobacco using Agrobacterium-mediated transient assay. Mol Biol Rep 36:1915–1921