Acta Physiol Plant (2011) 33:2041–2049
DOI 10.1007/s11738-011-0733-9

SHORT COMMUNICATION

Expression of selected heat shock proteins after individually applied and combined drought and heat stress

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Received: 13 September 2010 / Revised: 11 January 2011 / Accepted: 15 February 2011 / Published online: 8 March 2011

Abstract Drought and heat stress are among the abiotic factors causing the most severe damage on plant crops. Their combination is quite common in dry and semi-dry regions worldwide and little is known about its effect on heat shock protein (HSP) profile in wheat plants. The expression of four HSP genes (*Hsp* 17.8, *Hsp* 26.3, *Hsp* 70 and *Hsp* 101b) in *Triticum aestivum* L. plants subjected to individually applied water deprivation or high temperature and their combination was monitored via one-step RT-PCR analysis. Changes in the expression levels of small HSPs (smHSPs), HSP70 and HSP100 were established also by SDS-PAGE. The combination of drought and heat induced HSP expression more effectively than the individually applied stresses. The induction of HSPs displayed greater rate in the drought-tolerant wheat variety Katya than in the drought-sensitive cv. Sadovo. The results obtained in wheat plants suggested that the effect of separately applied drought and heat shock cannot be extrapolated to their combination.

Keywords Heat shock proteins · Drought · Heat stress · Combined drought and heat stress · Wheat · One step RT-PCR analysis

Communicated by P. Sowinski.

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Abbreviations

D	Drought
DH	Combined drought and heat stress
EDTA	Ethylendiaminetetracetic acid
H	Heat stress
HSP	Heat shock protein
PMSF	Phenylmethanesulfonyl fluorid
RLS and RSS	Respectively Rubisco large and small subunits
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Abiotic stress is the primary reason for farm production loss worldwide, occasioning average yield reductions of more than 50% for major crops (Larkindale and Knight 2002; Valliyodan and Nguyen 2006; Tayyar 2010), among which is bread wheat (*Triticum aestivum* L.). In nature, individual stresses, such as drought and high temperatures, usually do not occur separately (Howarth and Ougham 1993). Combined effect of water deficit and heat shock on cereals has not been studied in details and published data show that it differs from the sum of the separately conducted ones, and cannot be extrapolated and integrated between them (Mittler 2006; Sreenivasulu et al. 2007). Previous studies report that plant response to combined drought and heat (DH) stress differed from the reaction to other stresses such as pathogen attack, cold or salt (Hasegawa et al. 2000; Rizhsky et al. 2002; Mittler 2006; Jäger et al. 2008).

Plants react to stress (such as low and high temperatures, drought, salinity, flooding and cold) in a similar manner by production of HSPs—part of the cell-specific protection

(Ritossa 1962; Wang et al. 2004; Kotak et al. 2007; Timperio et al. 2008). They have been described as highly conserved polypeptides, which play an important role for survival under both normal and extreme conditions (Vierling 1991; Kregel 2002; Kotak et al. 2007). Most HSPs manifest strong cytoprotective effects and supply cellular homeostasis via maintaining proteins in their functional conformations, preventing aggregation of non-native proteins, refolding of denatured ones and removal of harmful polypeptides, arising during stress conditions (Timperio et al. 2008). HSP production is also an essential component of thermotolerance (Wang et al. 2004). The synthesis of HSPs is elevated above a threshold temperature. This temperature varies within different plant species. Induction of most HSPs occurs at 35°C (Burdon 1993). Wheat plants start to synthesize HSPs when tissue temperature exceeds 32–33°C (Vierling 1991; Skylas et al. 2002).

Heat shock proteins are assigned to five major families based on sequence homology and relative molecular weight: HSP100, HSP90, HSP70, HSP60 and smHSPs (Wang et al. 2004; Kotak et al. 2007). High-molecular-weight HSP, smHSP and HSP70 families fell within the scope of our study. The last mentioned stress proteins are major and highly conserved HSPs not only in plants but also in bacteria and animals (Timperio et al. 2008). Some members of this family are constitutively expressed and play an essential role in folding of nascent polypeptides while others are expressed during environmental stresses and are involved in refolding of non-native proteins (Vierling 1991; Wang et al. 2004).

HSP100 family includes proteins whose molecular weight ranges from 84 to 104 kDa (i.e. high-molecular-weight HSPs). They are essential in establishing thermotolerance in plants (Miernyk 1999; Queitsch et al. 2000; Gulli et al. 2007; Kotak et al. 2007) and are subject of our study as well. Some of HSP100 family members have been reported as constitutively expressed proteins in plants, while others are induced by different stresses. HSP100 members perform their anti-stress function preventing protein aggregation by interaction with smHSPs and in cooperation with HSP70 chaperone system (Miernyk 1999; Wang et al. 2004; Lee et al. 2005; Gulli et al. 2007; Kotak et al. 2007).

The most abundant set of HSPs in higher plants have molecular mass ranging from 15 to 40 kDa, and they are known as smHSPs. They belong to at least six different gene families, whose products are related to different cell compartments (Polenta et al. 2007) and all of these polypeptides function as molecular chaperones (Vierling 1991; Smykal et al. 2000). In contrast to HSP70, which are present in plant tissues both constitutively and under adverse environment, smHSPs are synthesized in response to stresses. However, it should be marked that smHSPs have been detected in tissues of control wheat plants, and also during particular developmental stages (Mansfield and Key 1987; Miernyk 1999; Kotak et al. 2007). SmHSPs prevent the aggregation of cellular proteins in co-operation with HSP70 and are important factors for acquiring stress tolerance and particularly thermotolerance (Vierling 1991; Wang et al. 2004).

The effect of combined DH (soil drought and heat shock) stress on expression of some members of smHSP, HSP70 and HSP100 families in *Triticum aestivum* is the subject of the paper. The plants were submitted to individual D (soil drought) and H (heat shock) stress and to combination of them in order to compare the results from different stressors. Severe, but recoverable 7-day drought (Demirevska et al. 2008) was applied to obtain the most informative differences from the treatments. Heat shock of 40°C for 5 h was chosen based on previously published data (Burdon 1986; Porter and Gawith 1999; Skylas et al. 2002). Two wheat varieties (Katya—drought resistant and Sadovo—less tolerant to drought) have been used in the present experiment.

To study gene expression in one non-model plant species like wheat semi-quantitative RT-PCR analysis of total mRNA was chosen because of its exquisite sensibility (Sreenivasulu et al. 2007; Hu et al. 2009; Kiselev and Dubrovina 2010). Expression of *Hsp* target genes was assessed with RT-PCR, performed with specific primer pairs. However, gene expression pattern alone appears insufficient for the description of one system (Timperio et al. 2008; Gygi et al. 1999). This was the reason to include supportive protein pattern analysis. Protein patterns were revealed via SDS-PAGE separation, based on dry weight, in order to eliminate the influence of plant material dehydration on the HSP profiles.

The aim of this study was to analyze the HSP response to individually applied soil drought or heat shock and their combination at molecular (semi-quantitative one-step RT-PCR) and biochemical (SDS-PAGE pattern) level in two winter wheat varieties differing in drought resistance.

Materials and methods

Plant material and growth conditions

Plants from two varieties of winter wheat (*Triticum aestivum* L.)—cv. Katya and cv. Sadovo were used. Cultivar Katya is recognized by the Institute of Plant Genetic Resources, Sadovo, South Bulgaria, as the most drought-resistant variety in Bulgaria and cv. Sadovo—disease-resistant (Simova-Stoilova et al. 2006, 2010). A preliminary screening confirmed that cv. Sadovo was less drought resistant compared with cv. Katya (Kalapos

et al. 1996; Simova-Stoilova et al. 2006, 2010; Demirevska et al. 2008). Plants were grown as soil cultures in plastic pots, containing 400 g of screened cinnamonic soil (pH 6.2) optimally fertilized with N, P and K. Wheat seedlings were grown at a day/night temperature regime of 22–25°C and the normal 50% air moisture. Illumination was provided by cool-white fluorescent lamps with photosynthetically active radiation of 200 μmol m^{-2} s^{-1} with 14-h photoperiod. Plants were daily watered to maintain 70% relative soil humidity (Demirevska et al. 2008) to the 8th day after the seed planting. On the 8th day, when the plants were with fully developed first and expanding second leaf, drought was imposed and irrigation was withheld for a period of 7 consecutive days. On the last date of this period the soil humidity reached 56–58% (Simova-Stoilova et al. 2006; Demirevska et al. 2008). Control plants and pots used only for the heat shock treatment were subjected to optimal water supply. Both, daily watered (14 days old) and 7 days drought-stressed wheat plants (14 days old) were incubated once at 40°C for 5 h in the middle of the day to obtain, respectively, only heat shocked or combined drought/heat shocked plants. Samples for the analyses were collected right after the application of the heat stress on 1st (C1, control plant material) and 7th (C7, control; D7, plant material from plants submitted to 7 days of water deprivation; DH7, plants subjected to 7 days of drought in combination with 40°C for 5 h and H7, only heat shocked daily watered wheat plants) days of the drought treatment, when wheat plants were, respectively, 8 and 14 days old. Leaf material was quickly frozen in liquid nitrogen and stored at −84°C for subsequent extractions. All analyses were performed with material derived from the fully developed first leaf. Data are obtained from three independent experiments.

Protein extraction and SDS-PAGE

Samples for the SDS-PAGE were prepared from fresh plant material, corresponding to 27 mg dry leaf material. Plant material was ground in liquid nitrogen and homogenized in 1 ml ice-cold 100 mM Tris–HCl buffer with pH 8, containing 20 mM $MgCl_2$, 2 mM EDTA (Ethylendiaminetetracetic acid), 20 mM β-mercaptoethanol, 2% Polyclar, 1 mM PMSF (Phenylmethanesulfonyl fluorid), 10% glycerol and 2% quartz sand. After centrifugation (30 min, 15,000 rpm, 4°C) the supernatant was boiled in sample buffer for SDS-PAGE. Leaf soluble proteins were separated by 12% SDS-PAGE, using a *Mini Protean II Dual Slab Cell* (BioRad system) according to Laemmli (1970). Samples loaded on each start contained equivalents of soluble protein extracted from 0.41 mg dry leaves. Dalton Mark Standard Mix (14–66 kDa, Sigma-Aldrich Chemie GmbH, Munich, Germany) and Precision Plus Protein Standards Dual Color (Bio-Rad) were used as a references. Gels were stained with Coomassie brilliant blue R-250.

Total RNA extraction

Total RNA was extracted from the first fully developed leaves with RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. The quality of the samples was checked on ethidium bromide-stained agarose gel, and RNA concentration was measured by assessing the absorption at 260 and 280 nm (UV-1601 spectrophotometer, Shimadzu).

One step RT-PCR analyses

Primers to amplify wheat *hsp* genes (AAK51797.1, a cytosolic class II small heat shock protein 17.8 kDa; nuclear gene encoding mitochondrial small heat shock protein 26.3 kDa; 70 kDa heat shock protein (TaHSP70d); heat shock protein 101b, Hsp101b) were designed using published *Triticum aestivum* mRNA sequences (http://www.ncbi.org) and are listed in Table 1.

The amplification products have the following predicted sizes: *Hsp17.8*, 417 bp; *Hsp26.3*, 339 bp; *Hsp70*, 456 bp; and *Hsp101b*, 200 bp.

RT-PCR analyses were performed with QIAGEN® OneStep RT-PCR Kit, using 20 ng total RNA per reaction, RNasin® Ribonuclease Inhibitor (Promega Corporation, Madison, USA) and gene-specific primers (Table 1), according to the manufacturer's instructions in PCR Mastercycler (Eppendorf). RT-PCR began with 30 min incubation of samples at 50°C—reverse transcription step. It has been followed by a conventional PCR: 1 cycle denaturation step at 95°C for 4 min; 25 cycles of denaturation/

Table 1 Specific *Hsp* primers used in one-step RT-PCR

Primers	NCBI gene bank Accession number
Hsp17.8	*AF350423*
(F) 5′-GCGGCCGCGAGAATGGAG-3′	
(R) 5′-GCGGCACACGGCGGAGAT-3′	
Hsp26.3	*AF104108*
(F) 5′-CATGGCCCGTCTGCTGTCTCT-3′	
(R) 5′-AGCACGCCGTTCTTCATCTCG-3′	
Hsp70	*AF005993*
(F)5′-CCCAGCGCCAGGCCACTAAGGAC-3′	
(R) 5′-CAAAGCGAGCCCGTGTGATGGTA-3′	
Hsp101b	*AF097363*
(F) 5′-TGGAGAGGAAGCGGATTCAG-3′	
(R) 5′-CTGCTTCAGCTTCCGGATCT-3′	

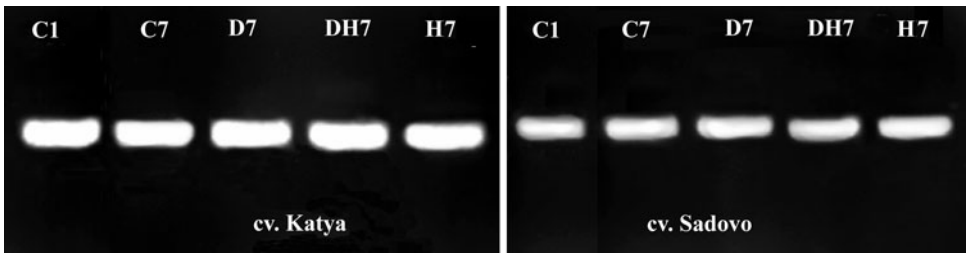


Fig. 1 RT-PCR amplification products of α-tubulin (as a reference gene) in the first fully expanded leaf of control (*C1*—1st and C7—7th day of stress, respectively, 8- and 14-day-old plants, daily watering) and stressed (*D7*—7 days drought-stressed 14-day-old plants, *DH7*—combined stress: heat-stressed D7-plants aged 14 days, H7—heat stress applied separately on 14-day-old plants) cv. Katya and cv. Sadovo wheat plants

annealing/elongation at 95°C for 30 s/61°C (or 64.5°C for Hsp17.8) for 30 s/72°C for 1 min followed by 1 cycle final extension at 72°C for 10 min.

Expression (Fig. 1) of *Triticum aestivum* α-tubulin gene (accession U76558, primers—(F) 5′-AGCGCCTTTGAGC CTTCGTCC-3′ and (R) 5′-TCATCGCCCTCATCACC GTCC-3′) under the same RT-PCR conditions (Ta = 61°C) was used as a reference to normalize *Hsp* expression data.

Data processing and statistical analysis

SDS-PAGE was performed many times with samples from three independent protein extractions. The resulting gel images were similar. Representative images of the obtained protein profiles are shown on Fig. 2. All gels were scanned and collected data were processed with ImageJ 1.41ov software. Results are expressed as total area in percentage occupied by each band and vertical bars indicate the

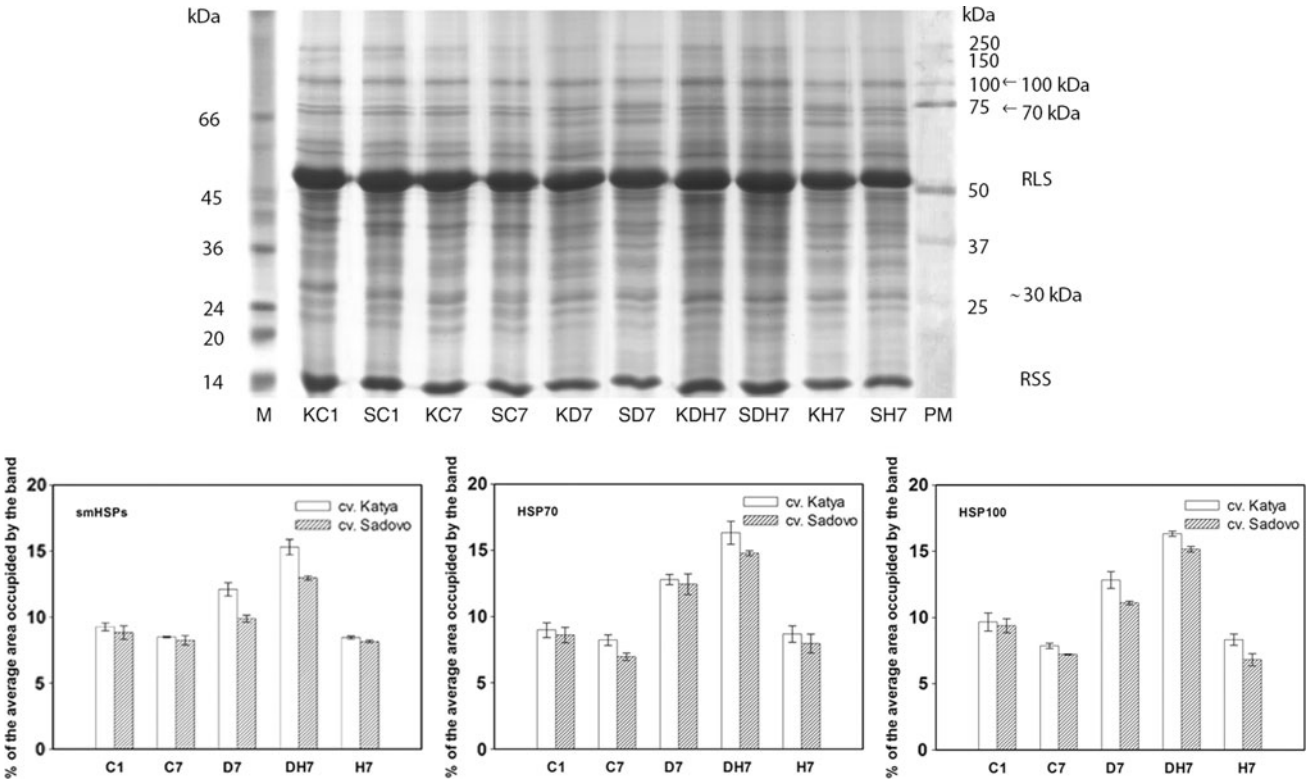


Fig. 2 Leaf protein pattern after 12% SDS-PAGE separation of extracts derived from leaves of cv. Katya (*K*) and cv. Sadovo (*S*) controls *C1* (1st day of drought stress- respectively, 8-day-old plants, daily watering) and *C7* (7th day of drought stress- respectively, 14-day-old plants, daily watering), *D7* (7 days drought-stressed, respectively, 14-day-old plants), *DH7* (the same as D7, additionally heat shocked for 5 h at 40°C in the sampling day), *H7* (only heat stressed for 5 h at 40°C 14-day-old plants). Dalton mark standard mix (*M*) and Precision plus protein standards dual color (*PM*) have been loaded, respectively, on the *first* and the *last lanes*. Positions of Rubisco subunits (RLS and RSS) and the molecular weights of studied HSPs are indicated as *reference points* to the right

standard error (calculated with SigmaPlot for Windows, Version 9.00).

Three independent RT-PCR analyses were performed and the reactions were loaded on ethidium bromide-stained 1% agarose gels for visualization. Gel images show results from one representative experiment. Quantification of bands corresponding to the amplification products was performed with ImageJ 1.41ov software. Data were expressed as a total area in percentage occupied by each band. Graphs depict target *Hsp* gene and α-tubulin expression ratio from the three independent experiments and the vertical bars indicate the standard error (calculated with SigmaPlot for Windows, Version 9.00).

Results

Wheat cultivars Katya (drought resistant) and Sadovo (less drought tolerant) were subjected to three different stresses—water deprivation, heat shock and combination of them. Our preliminary studies confirmed that a drought period of 7 days produced the most representative and significant results regarding changes in stress-protein content and other physiological parameters. Demirevska et al. (2009) determined the degree of water deprivation by analyzing changes in growth parameters of wheat plants and by determining the water deficit from leaf material taken from the same two varieties. In our previous paper it was also established that relative water content of wheat leaves decreased significantly to 24.5% for cv. Sadovo and to 29% for cv. Katya on the 7th day of D, combined with heat shock. Relative electrolyte leakage of ions markedly increased to 67.9% on the same day (Grigorova et al. 2011).

The expression of four HSP genes (Fig. 3) in wheat plants of the two tested varieties (Katya and Sadovo) subjected to 7 days of drought, high temperature (40°C for 5 h) and their combination was assessed by semi-quantitative one-step RT-PCR analysis. Images on Fig. 4a–d are from representative experiments. The low-molecular-weight HSPs (17.8 and 26.3) were highly abundant in samples derived from both drought-stressed (D7) and control (C7) plants. The most important observation was that *Hsp17.8* expression in the C1 control samples of the tolerant variety was almost four times higher than the expression in the less tolerant Sadovo (Fig. 4a). *Hsp26.3* transcript levels did not change significantly after stress treatments (Fig. 4b). *Hsp70* transcript levels increased twice and even more than twice in plants submitted to combined and heat stress (DH7 and H7) in comparison with the control plants from the same day (see Fig. 4c). *Hsp101b* increased in stressed leaves submitted to simultaneously applied drought and heat stress (DH7) and in plants submitted to individual heat shock (H7) (see Fig. 4d).

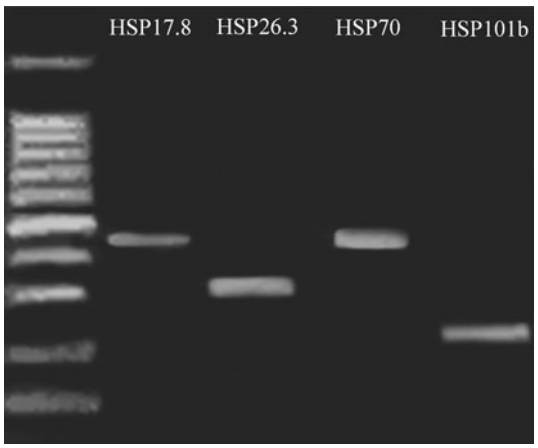


Fig. 3 PCR amplification products of the studied *Hsp* genes

The obtained protein profile via 12% SDS-PAGE loaded on dry weight base (Fig. 2) gave us an important information about the expression of the soluble proteins. Bands corresponding to low- as well as high-molecular-weight HSPs (with mass around and above 100 kDa) and HSP70 exhibited the most consistent alterations during examined stress factors. The mentioned proteins were detected with Dalton Markers and by immunoblot analyses with polyclonal antibodies against Hsp110 and αβ-crystalin and monoclonal antibodies against Hsp70 (Grigorova et al. 2011). Diagrams under the gel picture on Fig. 2 showed the differences in expression levels of proteins with molecular weights around 30-, 70- and 100-kDa, respectively, recognized as αβ-crystalin (a member of the smHSP with molecular mass around 30 kDa), Hsp70 and Hsp100. Small Hsp (in case Hsp30) exhibited rates almost twice higher compared with respective control plants (C7) during combined stress (DH7). Detected differences during individually applied water deprivation (D7) were less pronounced—increase between 20 and 40% in the different examined wheat varieties was detected. The tolerant cv. Katya exhibited around 20% increase in smHSP expression compared with the more sensitive cv. Sadovo during D and DH stresses.

Similar trend has been observed in HSP100 expression (see Fig. 2). Combined stress (DH7) provoked 100% elevation, while drought (D7) resulted in 60% increase compared with the control plants (C7). HSP100 expression was least influenced by the separately applied heat shock (H7) (Fig. 2). The tolerant cv. Katya had higher high-molecular-weight HSPs expression under individual D stress and combined DH stress compared with the less tolerant cv. Sadovo—around 16 and 7%, respectively.

Hsp70 increased more than 100% under DH conditions according to SDS-PAGE separation. Individually applied drought and heat stress resulted in 60% (D7) and 10% (H7)

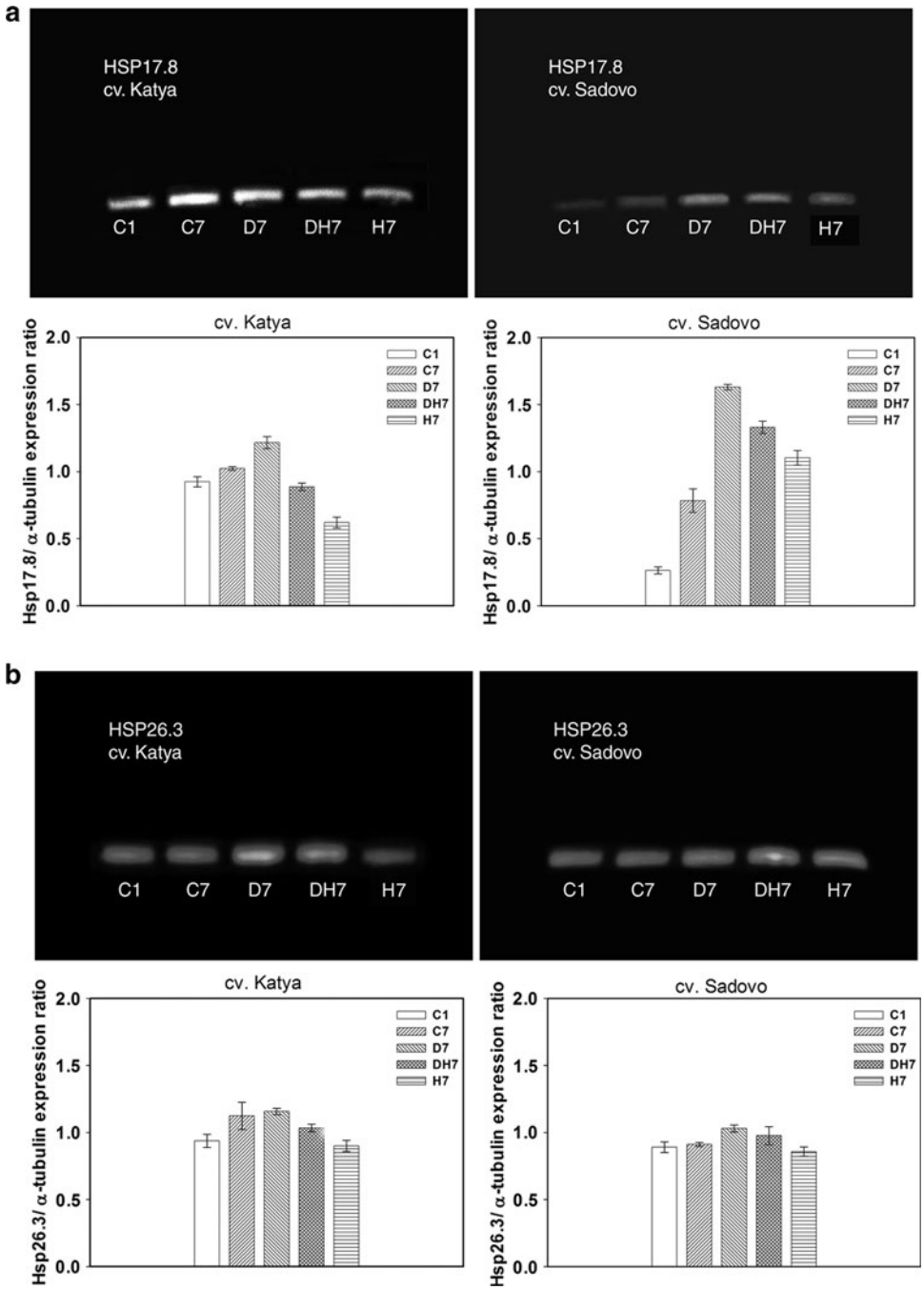


Fig. 4 RT-PCR amplification products of *Hsp17.8* (**a**), *Hsp26.3* (**b**), *Hsp70* (**c**) and *Hsp101b* (**d**) in the first fully expanded leaves of cv. Katya and cv. Sadovo wheat plants at different conditions: *C1*, control of 1 day of drought or 8-day-old plants (daily watering); *C7*, control of 7 days of drought or 14-day-old plants (daily watering); *D7*, 7 days of drought or 14-day-old plants; *DH7*, combined stress: heat-stressed D7-plants; *H7*, only heat stress applied on 14-day-old plants (daily watering). Diagrams show relative expression ratio of the respective *Hsp* and α-tubulin. *Vertical bars* indicate the standard error

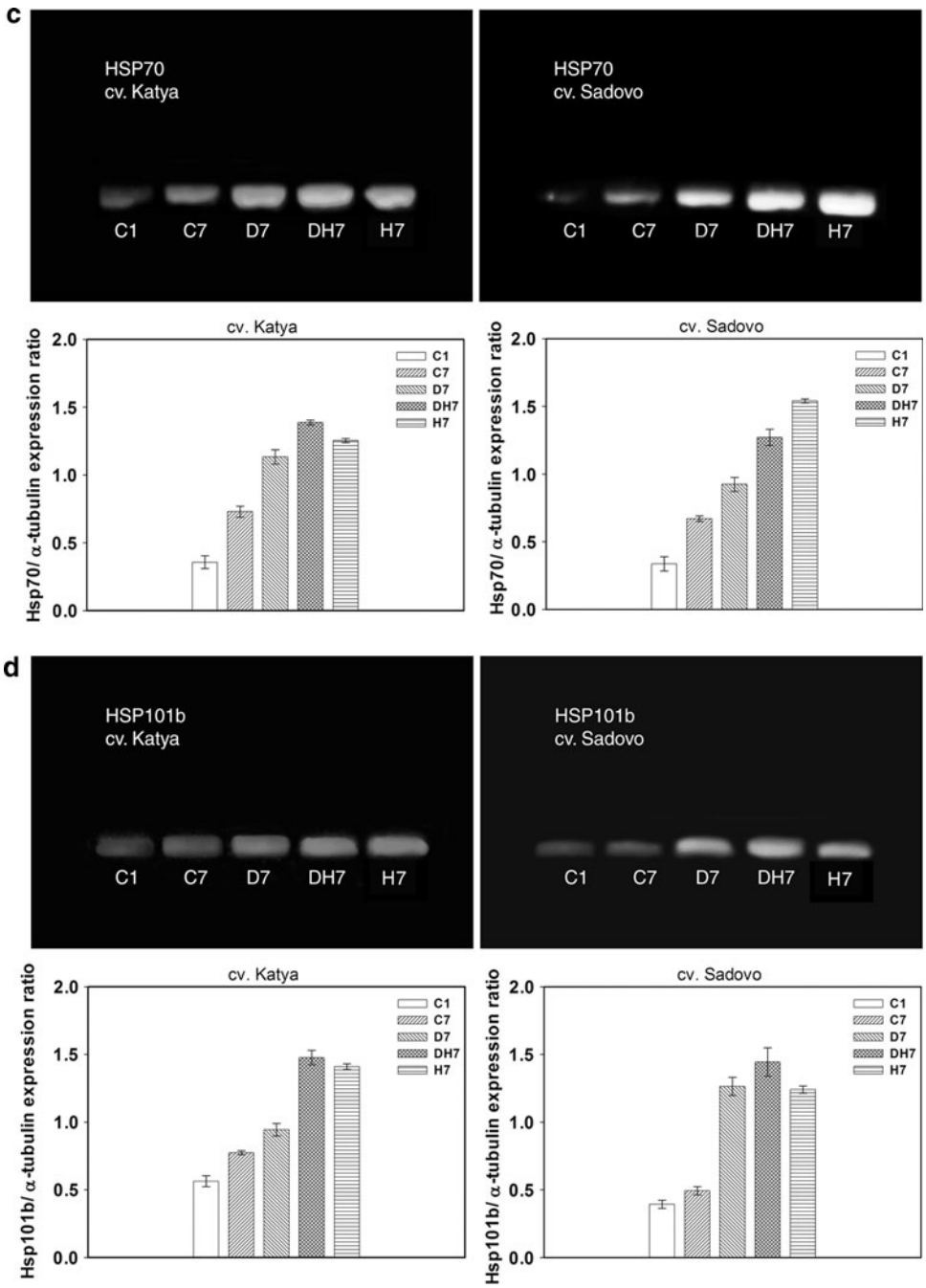
increase in Hsp70 expression. Differences between cv. Sadovo and cv. Katya were established also in Hsp70 profile. The drought-tolerant variety Katya had higher Hsp70 expression during DH and D stress with 20 and 3%, respectively.

Each crop may react differently under stress conditions. The comparative analysis of HSP expression, during drought, high temperature and their combination in wheat showed that the combined stress was the most destructive to plants. HSP response to drought resembles the one observed during combined stress and differs significantly from the response obtained after individually applied heat shock. Synthesis of HSP in resistant wheat variety is stronger than in the sensitive one, and this differs wheat from tobacco and *Arabidopsis* plants (Rizhsky et al. 2002, 2004).

Discussion

High temperature and reduced water supply have high impact on plant productivity and they are serious threats to

Fig. 4 continued



agriculture, because usually they lead to protein dysfunction (Howarth and Ougham 1993; Sreenivasulu et al. 2007; Timperio et al. 2008). Chaperone activity of HSPs defines their basic role in the protein turnover during stress conditions (Burdon 1993). On the other hand, little is known about the influence of combined drought/heat stress on HSP expression pattern (Rizhsky et al. 2002; Rizhsky et al. 2004; Mittler 2006). Our work presents a comparative study of changes in certain heat shock protein profiles (smHSPs, HSP70 and HSPs with high molecular weight) during combined and individually applied drought and heat stress in *Triticum aestivum* L., using the potentials of gene and polypeptide pattern approaches.

Rizhsky et al. (2002, 2004) previously found in *Arabidopsis* and tobacco considerable increase in HSP level under DH stress in comparison with separately applied D and H stress, with a major contribution of smHSPs. Our previous study (Grigorova et al. 2011) with wheat plants, submitted to individually applied D and H, and their combination confirmed the tendency of highest HSP

expression profiles during DH stress but using only immunoblot analysis. In the present work, an increase in *Hsp*70 transcripts after DH and H stresses has been detected. Hsp70 is considered as a major molecular chaperone, which is involved in plant stress response (Vierling 1991; Wang et al. 2004) and its over-expression in plants, especially during DH treatment, represents an incontestable evidence for its great protective role. The highest levels of HSP expression are also proof for the strongest disruptive effect of combined DH stress in wheat plants. Hsp70 had stronger expression in the tolerant cv. Katya compared with the sensitive cv. Sadovo, especially during DH stress, at both gene expression and protein level. Similarly, Vassileva et al. (2009) detected striking differences between the two cultivars at cell morphology level. These suggest that HSPs represent an important group of the stress-adaptive set in the tolerant crop varieties.

High-molecular-weight HSPs also fell within the scope of the present study. The highest content of *Hsp101b* transcripts was observed under DH treatment. These results correspond to the major protective role of the high-molecular-weight HSPs against protein aggregations (in collaboration with Hsp70 chaperones), under severe DH conditions, reported previously by Queitsch et al. (2000) and by Wang et al. (2004). The comparatively higher level of *Hsp101b* documented during H stress confirmed the recent published data about the participation of Hsp101 in the establishing of thermotolerance in *Arabidopsis* plants (Queitsch et al. 2000).

RT–PCR analyses of *Hsp26.3* and *Hsp17.8* showed significant expression under severe drought, which supported their chaperon activity (Smykal et al. 2000; Kotak et al. 2007). Experimental data also documented that *Hsp26.3* and *Hsp17.8* transcripts were highly represented in control plants. Their accumulation could be provoked either by adverse environment or by developmental factors (Vierling 1991; Waters et al. 1996; Smykal et al. 2000), which explains their high levels in the leaves of the plants at early vegetation stage (8–14 days after germination). The significantly higher expression of the small *Hsp17.8* in control plants of the tolerant cv. Katya compared with less tolerant cv. Sadovo probably is an acquisition related to its better potential to cope with water deficit. SDS-PAGE separation showed highest levels of smHsps in DH stress, followed by the drought-treated plants. Gene expression results alone are not sufficient to reflect the actual changes in the protein levels in vivo, due to the following post-transcriptional changes and posttranslational modifications of the polypeptides under stress conditions (Gygi et al. 1999; Timperio et al. 2008; Hassan et al. 2010; Simova-Stoilova et al. 2010).

Our results show that severe stresses like D, H and combined DH provoke higher expression of the examined HSPs in drought-tolerant variety Katya, which corresponds with its higher adaptive possibilities, and that the responsiveness of HSPs to drought and heat stress, and their important cellular function make them potential biochemical markers for assessment of heat, drought or heat/drought resistance (Alvim et al. 2001; Rizhsky et al. 2002).

In conclusion, alterations in protein expression provoked by drought seemed similar to these induced by DH stress, while the observed HSP changes under individually applied heat shock were significantly different. Drought resistant cv. Katya showed an up-regulated HSP expression during D and DH stress in comparison with the less tolerant cv. Sadovo. Drought/heat stress induced the highest HSP expression in wheat plants compared with separate D and H. Thus, our study confirmed the observation of Rizhsky et al. (2002, 2004) in *Arabidopsis* and tobacco that the combination of drought and heat stress alters plant metabolism in a different manner compared with single stresses, and that established changes under separated stressors cannot be just summed to predict their combined effect on the crops.

Acknowledgments This study was financially supported by SCOPES program of the Swiss National Science Foundation (project DILPA–JRP–IB73AO-111142/1) and the Ministry of Education and Science of Republic Bulgaria (Contract N: CC1503). We are grateful to Mrs. B. Juperlieva-Mateeva, Mrs. A. Kostadinova and Mrs. I. Anders for their technical assistance.

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