Combined drought and heat stress in wheat: changes in some heat shock proteins

B. GRIGOROVA[1]*, I. VASEVA[1], K. DEMIREVSKA[1] and U. FELLER[2]

Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Building 21, BG-1113 Sofia, Bulgaria[1]
Institute of Plant Sciences and Oeschger Centre for Climate Change Research, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland[2]

Abstract

The influence of combined and individually applied drought and heat stress was studied in two wheat (*Triticum aestivum* L.) cultivars: resistant cv. Katya and susceptible cv. Sadovo. Relative water content decreased and electrolyte leakage increased due to individual and combined application of both stresses. Initial heat shock protein profile has been outlined *via* SDS electrophoresis of leaf extracts. The results obtained were confirmed by immunoblotting with anti-HSP70 monoclonal antibodies, anti-HSP110 polyclonal antibodies and anti-αβ-crystalline polyclonal antibodies. The effect of simultaneously applied water stress and heat shock resembled the alterations in protein expression provoked only by water stress and differed significantly from the changes occurring after the individual application of heat stress.

Additional key words: electrolyte leakage, immunoblotting, relative water content, *Triticum aestivum*.

Introduction

Plants in the field are frequently subjected to abiotic stresses that affect adversely their growth, development and productivity (Chaves *et al.* 2004, Kotak *et al.* 2007). They developed different mechanisms to respond to external conditions (Vierling 1991, Waters *et al.* 1996, Schoffi *et al.* 1998, Lee *et al.* 2000, Smykal *et al.* 2000, Ferguson 2004, Wang *et al.* 2004). Drought and heat stress are among the factors causing the most severe damage (Wang *et al.* 2004, Sumesh *et al.* 2008, Santos *et al.* 2009). Each of these stresses has been extensively studied but little is known about their combined impact on wheat plants (Kregel 2002, Rizhsky *et al.* 2002, 2004, Mittler 2006). The combination of high temperature and water deficit is quite common in dry and semi-dry regions across the world and claims extensive agricultural losses (Mittler *et al.* 2001, Rizhsky *et al.* 2002, Moffat 2002, Kotak *et al.* 2007, Sumesh *et al.* 2008).

Similar responses to combined drought and heat stress have been described in *Arabidopsis* and tobacco (Rizhsky *et al.* 2002, 2004). It was found out that a combination of drought and heat stress provokes cessation of conventional protein synthesis, accompanied by increased translation of heat shock proteins (HSPs) and other stress related proteins (Vierling 1991, Schoffi *et al.* 1998, Mittler 2006, Caeiro *et al.* 2008, Lin *et al.* 2008).

HSPs were first discovered in 1962 and described as a set of proteins with expression induced by heat shock and a variety of other stresses (Ritossa 1962, Wang *et al.* 2004). They have been described as highly conserved polypeptides which play an important role for survival under both normal and extreme conditions (Vierling 1991, Schoffi *et al.* 1998, Kregel *et al.* 2002, Kotak *et al.* 2007). HSP production is an essential component of thermotolerance (Vierling 1991, Schoffi *et al.* 1998,

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Abbreviations: D - drought stress; DH - combined drought and heat stress; EDTA - ethylendiaminetetracetic acid; H - heat stress; HSP - heat shock protein; PMSF - phenylmethanesulfonyl fluoride; RLS and RSS - Rubisco large and small subunits, respectively; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis.
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* Corresponding author; fax: (+359) 2 8739952, e-mail: biligi@mail.bg

Kregel *et al.* 2002, Wang *et al.* 2004). Wheat plants begin to synthesize HSPs when tissue temperatures exceed 32 - 33 °C (Vierling 1991). Plant HSPs have been classified in five major groups: HSP100, HSP90, HSP70, HSP60, and small HSPs (smHSPs) based on their molecular mass (Wang *et al.* 2004, Kotak *et al.* 2007).

HSP100 are members of the large AAA family of ATPases and play a central role in establishing thermotolerance in plants (Miernyk 1999, Queitsch *et al.* 2000, Kotak *et al.* 2007, Gulli *et al.* 2007). They are involved in the response to different stresses because these proteins are able to interact with smHSPs (Lee *et al.* 2005), and also to prevent protein aggregation at high temperature in cooperation with the HSP70 chaperone system (Miernyk 1999, Wang *et al.* 2004, Kotak *et al.* 2007, Gulli *et al.* 2007). Members of the HSP60 family function as molecular chaperones. Their major role is thought to involve protein assembly (Vierling 1991, Wang *et al.* 2004). HSP70 proteins are a highly conserved, ATP-dependent ubiquitous set of molecular chaperones. Some members of this family are expressed constitutively, while others are expressed during environmental stresses and are involved in refolding of non-native proteins (Vierling 1991, Miernyk 1999, Wang *et al.* 2004, Mayer *et al.* 2005).

HSP110 is a diverged relative of the HSP70 family and is considered as a subfamily of the HSP70 on the basis of similarities in structural and functional properties, but possessing certain genetic uniqueness (Oh *et al.* 1999, Wang *et al.* 2004). HSP110 are able to prevent protein aggregation and maintain denatured protein in a soluble (folding-competent) state, but with greater capacity as compared to HSP70. Their over-expression correlated with thermotolerance *in vivo* (Oh *et al*. 1999, Sumesh *et al.* 2008). In plants, HSP110 synthesis is more transient than that of other HSPs. It is primarily limited to the 1st hour of heat stress (Vierling 1991).

Small HSPs have molecular mass from 15 to 30 kDa (Waters *et al.* 1996, Smykal *et al.* 2000). Higher plants synthesize predominantly (up to 1 % of the total proteins) and ubiquitously smHSPs in response to stress, which function as molecular chaperones (Vierling 1991, Miernyk 1999, Smykal *et al.* 2000, Wang *et al.* 2004). In contrast to HSP70 and HSP60, which are present in plant tissues both constitutively and under adverse environmental impacts, smHSPs are synthesized basically in response to stresses and some of them are also expressed during certain developmental stages (Waters *et al.* 1996, Miernyk 1999, Smykal *et al.* 2000, Wang *et al.* 2004, Kotak *et al.* 2007). It should be borne in mind that smHSPs were detected not only under stress conditions but also in the tissues of control wheat plants (Mansfield 1987). All these characteristics define smHSPs as important factors in acquiring stress tolerance and particularly in the development of thermotolerance (Vierling 1991, Waters *et al.* 1996, Wang *et al.* 2004). It was proved that αβ-crystalline, which is a member of the smHSP family, prevents aggregation of proteins (Santhosh Kumar and Sharma 2006).

The earlier studies have shown that plant response to combined drought/heat (DH) stress differed from the reaction to other stresses, such as cold or salt stress or pathogen attack (Schoffi *et al.* 1998, Rizhsky *et al.* 2002, Mittler 2006). Generally, HSPs induction in plants is higher under combined drought and heat stress, as compared to the induction observed under individually applied heat shock or drought. Comparatively higher expressions of smHSPs, HSP70 and HSP100 were established during DH. The most prominent increase was reported in the content of HSP18 in tobacco and *Arabidopsis* plants subjected to DH (Rizhsky *et al.* 2002, Rizhsky *et al.* 2004).

In view of the insufficient information on DH stress effect on *Triticum aestivum* and about the role of the above-described proteins in stress response, a specific and detailed study of the expression of selected members of smHSPs, HSP70 and HSP100 families was initiated under drought (D), heat shock (H) alone and DH in resistant and susceptible wheat cultivars. The relative water content (RWC) and electrolyte leakage (EL) in differently stressed wheat plants were monitored to evaluate physiological changes during DH stress, as compared to individually applied conditions.

Materials and methods

Wheat (*Triticum aestivum* L.) cv. Katya, agriculturally characterized (at the Institute of Plant Genetic Resources, Sadovo, South Bulgaria) as the best drought-tolerant Bulgarian cultivar, and less tolerant cv. Sadovo were used for the experiments (Kalapos *et al.* 1996, Simova-Stoilova *et al.* 2006, Demirevska *et al.* 2008). Plants were grown in a growth chamber, with temperature of 22 - 25 °C and photosynthetically active radiation of 150 µmol m^{-2} s^{-1} during a 14-h photoperiod in pots containing 400 g of leached cinnamon soil, pH 6.2, 8 mg(N) kg^{-1}, 13.2 mg(P) kg^{-1} and 100 mg(K) kg^{-1} (Geneva *et al*. 2006). Additionally, they were optimally fertilized with N, P and K and watered daily to maintain 70 % of soil moisture (Demirevska *et al.* 2008). Drought stress was applied to 8-d-old plants (plants with fully developed first and expanding second leaf) by withdrawing water for 7 consecutive days until soil moisture reached 56 - 58 % (Simova-Stoilova *et al.* 2006, Demirevska *et al.* 2008). Control plants were irrigated daily to maintain 70 % soil moisture. Differently stressed plants recovered for 3 d after rehydration. Samples were collected simultaneously from the first fully developed leaf of the controls (C1 - 8-d-old daily watered plants and C7 - 14-d-old daily watered plants) and stressed plants D1, D5, D6, D7 (1, 5, 6, or 7 d drought stressed plants); as well as from DH5, DH6, DH7 (the same as D plants,

but additionally heat shocked for 5 h at 40 °C during the sampling day), H5, H6, H7 (12, 13, 14-d-old, daily watered plants, heat shocked for 5 h at 40 °C during the sampling day), and R5, R6, R7 (5, 6 or 7 d stressed plants recovered 3 d after rehydration, which corresponded to 16-, 17- and 18-d-old plants.

Leaf relative water content (RWC) was determined according to Barrs and Weatherley (1962): RWC = [(FM - DM)/(TM - DM)] × 100, where FM is the fresh mass of leaves, TM is the fully water-saturated leaf mass after soaking for 24 h in distilled water at 4 °C, and DM is the dry mass measured after 5 h at 104 °C.

Membrane integrity was evaluated by relative electrolyte leakage (EL) of 2 cm leaf segments floating on distilled water for 24 h at 4 °C (using a conductivity meter), and expressed in percentage of the total leaf electrolyte content obtained after boiling the segments (Nunes and Smith 2003).

Samples for the SDS-PAGE and immunoblot analyses were prepared from 150 mg fresh leaves frozen in liquid nitrogen prior to extraction. The samples were ground to powder with liquid nitrogen and were extracted by 1 cm^3 ice-cold 100 mM Tris-HCl buffer (pH 8) containing 20 mM $MgCl_2$, 2 mM EDTA, 20 mM β-mercaptoethanol, 2 % *Polyclar*, 1 mM PMSF, 10 % glycerol, and 2 % quartz sand. After centrifugation (30 min, 15 000 *g*, 4 °C) the supernatant was boiled in sample buffer for SDS-PAGE. The total soluble protein content was measured according to the method of Bradford (1976) at 595 nm, with bovine serum albumin as a standard.

Leaf soluble proteins were separated by 12 % SDS-PAGE, using a *Mini Protean II Dual Slab Cell* (*BioRad*, Hercules, USA), according to Laemmli (1970). Samples containing equivalents of soluble protein extracted from 5 mg(FM) were loaded at all starts. *Dalton Mark* standard mix (14 - 66 kDa, *Sigma*) was used as a reference. The seven replicates of SDS-PAGE electrophoreses have produced similar profiles. One of them was selected to visualize the obtained protein pattern. Gels were stained with Coomassie brilliant blue R-250 and their scanned images were analyzed with *ImageJ* software version *1.41*.

Immunoblot analyses were performed with samples initially separated on 12 % SDS-PAGE. Soluble proteins were transferred to a nitrocellulose membrane, as described by Mitsuhashi and Feller (1992), using the *Trans Blot* system (*BioRad*). *Precision Plus* protein standards dual color (*Bio-Rad*) molecular mass markers were loaded as a reference. The membranes were blocked for 2 h in TTBS buffer (0.1 M Tris, pH 7.9, 0.15 M NaCl, 0.1 % *Tween 20*) containing 1 % ovalbumin, and probed with anti-HSP110 (*Stressgen*, Canada), anti-αβ-crystalline polyclonal antibodies (developed at the Institute of Immunology, Bulgarian Academy of Sciences, Sofia, Bulgaria) and anti-HSP70 monoclonal antibodies (*Stressgen*). Goat anti-mouse IgG was used as secondary antibody for HSP70 detection. Detection of HSP110 and αβ-crystalline was accomplished by goat anti-rabbit IgG as secondary antibody. PAP-complex (peroxidase-anti-peroxidase) was used to enhance the sensitivity of the antigen-antibody reactions. The bands were visualized with 4-chloro-α-naphtol (*Sigma*).

RWC and EL data were measured in three replicates (each replicate comprised measurements of the parameter in nine leaves). The results were statistically processed with *SigmaPlot for Windows*, version *9.00*. SDS-PAGE was performed seven times and immunoblottings three times. The resulting gel images were similar. Representative images of the obtained protein profiles are shown. All gels were scanned and the obtained data were processed with *ImageJ*, version *1.41* software for quantification of bands.

Results

Wheat plants grown under controlled conditions were submitted to drought and heat stress, separately and in combination, for 5, 6, and 7 consecutive days, and each treatment group recovered for 3 d. Preliminary studies

Table 1. Relative water content, RWC [%] measured in the first fully expanded leaf of differently treated (C - unstressed, D - drought, DH - drought and heat, H - heat) wheat plants (cvs. Katya and Sadovo) during the experiment from day 1 (D1) to day 7 (D7). Means ± SE, *n* = 3

Cultivar	Treatment	D1	D2	D3	D4	D5	D6	D7
Katya	C	92.8 ± 0.32	97.2 ± 0.46	95.4 ± 1.18	90.8 ± 0.14	92.9 ± 0.22	97.6 ± 0.20	95.2 ± 0.66
	D	94.6 ± 0.36	91.3 ± 0.54	93.2 ± 0.50	90.8 ± 0.14	75.4 ± 0.32	46.9 ± 0.78	38.1 ± 0.46
	DH					42.1 ± 0.15	35.2 ± 0.59	29.0 ± 0.89
	H					95.3 ± 0.70	91.1 ± 0.17	88.9 ± 0.13
Sadovo	C	93.4 ± 0.65	94.4 ± 0.63	94.3 ± 0.66	93.3 ± 0.56	97.9 ± 0.09	94.8 ± 0.60	92.9 ± 0.04
	D	93.1 ± 0.20	95.0 ± 0.07	93.1 ± 0.21	90.0 ± 0.09	90.2 ± 0.41	51.6 ± 0.53	36.9 ± 0.21
	DH					41.2 ± 0.49	33.7 ± 0.37	24.5 ± 0.26
	H					94.0 ± 0.58	85.7 ± 0.81	90.5 ± 0.87

Table 2. Electrolyte leakage, EL [%] measured in the first fully expanded leaf of differently treated (C - unstressed, D - drought, DH - drought and heat, H - heat) wheat plants (cvs. Katya and Sadovo) subjected for 5 d (D5), 6 d (D6) and 7 d (D7) to drought, with a subsequent 3 d recovery phase (R5, R6 and R7). Means ± SE, *n* = 3.

Cultivar	Treatment	D1	D5	D6	D7	R5	R6	R7
Katya	C	3.9 ± 0.15	4.1 ± 0.12	6.5 ± 0.12	7.6 ± 0.20	3.1 ± 0.09	11.4 ± 0.31	13.9 ± 0.54
	D		12.4 ± 0.12	26.1 ± 0.56	52.6 ± 0.81	3.3 ± 0.16	20.4 ± 0.35	25.8 ± 0.12
	DH		12.2 ± 0.11	53.5 ± 0.27	56.3 ± 0.17	3.7 ± 0.12	24.5 ± 0.17	31.1 ± 0.23
	H		3.6 ± 0.12	6.4 ± 0.12	6.9 ± 0.06	3.6 ± 0.12	9.1 ± 0.06	11.9 ± 0.17
Sadovo	C	3.1 ± 0.12	4.8 ± 0.17	5.9 ± 0.17	6.8 ± 0.17	2.9 ± 0.12	9.9 ± 0.03	9.9 ± 0.00
	D		13.6 ± 0.12	22.9 ± 0.12	54.4 ± 0.35	3.3 ± 0.06	17.2 ± 0.12	19.2 ± 0.12
	DH		16.9 ± 0.17	67.0 ± 0.12	67.9 ± 0.17	3.7 ± 0.12	23.9 ± 0.17	25.6 ± 0.23
	H		4.1 ± 0.17	5.7 ± 0.12	7.7 ± 0.17	3.6 ± 0.12	7.3 ± 0.17	10.4 ± 0.23

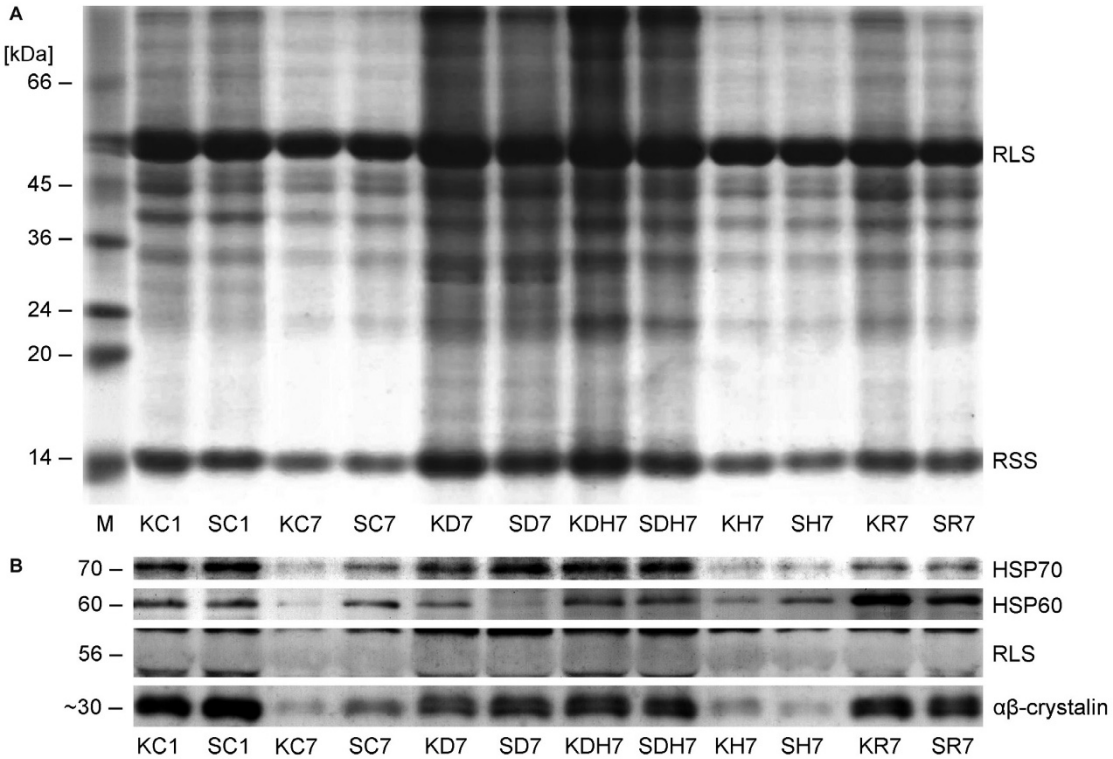

Fig. 1. Protein pattern after 12 % SDS-PAGE separation (*A*) and immunoblot of leaf heat shock proteins (*B*) of extracts derived from leaves of cv. Katya (K) and cv. Sadovo (S) controls - C1 (8-d-old plants, daily watered) and C7 (14-d-old plants, daily watered), D7 (7 d drought-stressed, 14-d-old plants), DH7 (the same as D7, additionally heat shocked for 5 h at 40 °C during the sampling day), H7 (only heat stressed for 5 h at 40 °C, 14-d-old plants), and R7 (recovered after 3 d irrigation DH7 - plants, 18-d-old plants). *Dalton Mark* standard mix (M) has been loaded on the first lane and the positions of Rubisco subunits (RLS and RSS) are indicated as reference points to the right in the SDS-PAGE profile.

confirmed that a 7-d stress produced the most representative and significant results regarding the obvious changes detected in stress-protein contents, RWC and EL. Electrophoretic analyses of plants submitted to drought for 8 d showed visible fragmentation of the Rubisco large subunit (data not shown), which outlines the limit between recoverable and lethal stress in wheat seedlings.

RWC remained practically unchanged during the first 4 d (for cv. Katya) and during 5 d (for cv. Sadovo) of water deprivation; afterwards it decreased significantly to 24.5 % for cv. Sadovo and to 29 % for cv. Katya on the 7th day of drought combined with heat shock (Table 1). Relative electrolyte leakage (EL) of ions, which stands evidence to the level of cell membrane integrity, markedly increased (67.9 %), especially on the 7th day of drought combined with heat stress (Table 2). The differences between the two tested wheat cultivars

regarding RWC and EL were not significant.

SDS-PAGE data (Fig. 1*A*) demonstrated that RLS was a constantly expressed protein with Mr of approximately 56 kDa. The processing of all applied gels showed a significant increase of its content, about 2.5 times as compared to the controls, under drought and drought/heat combination. Applied alone, heat stress did not provoke any changes and the protein pattern remained identical to the control one. Drastic increase in HSP70 expression was observed in plants subjected to drought, more than eightfold, and in drought/heat stressed plants almost tenfold, as compared to the relevant controls. HSP70 content remained higher in recovered DH7 plants submitted to combined stress, as compared to the respective controls, especially in the tolerant cv. Katya. This cultivar had a stronger HSP70 expression under D and DH, as compared to cv. Sadovo. However, heat stress alone did not influence HSP70 content. A similar trend was documented for HSPs with high Mr (above 100 kDa). The content of these proteins in cv. Katya was significantly higher then in cv. Sadovo. As expected, HSPs with Mr below 30 kDa, were translated at extremely high levels under drought and combined stress, reaching the highest content in cv. Katya under combined stress. A specific band migrating at a position close to the 30 kDa marker band was revealed only in samples derived from drought-stressed plants.

Immunoblot analyses of HSP110, HSP70 and αβ-crystalline protein (Fig. 1*B*) confirmed the initially obtained results from SDS-PAGE. HSP70 showed constitutive presence, with at least thrice higher levels in D, DH and the 1st day control samples, as compared to 7 d controls. Immunodetection of low Mr proteins was significantly enhanced in D, DH and R (recovered after 7 d drought in combination with heat stress) plants, as compared to the ones detected in the 7 d controls. Along with this, heat stress alone did not influence the smHSP contents. Anti-HSP110 immunoblotting resulted only in scarcely visible trace reactions, which could not produce an informative scanned image. Nevertheless, as it has been referred to in the anti-HSP110 antibody passport, a cross reaction localized near the position corresponding to HSP60 chaperone system was immunodetected. A stronger expression was observed in DH and R7 samples.

Discussion

This comparative study on drought and heat stress (applied separately and in combination) confirmed their influence on RWC, EL and protein composition in correspondence with previous results (Jiang *et al.* 2002, Rizhsky *et al.* 2002, 2004, Mittler 2006, Demirevska *et al.* 2008). In order to elucidate the distinct alterations in protein contents induced by different kind of stresses, severe but recoverable drought stress was applied (water deprivation for up to 7 d). When prolonged by another day, *i.e.* 8 d of drought stress, fragmentation of RLS (data not shown) was observed – a sign for initiated destructive changes in plants. According to Vierling (1991), temperature of 32 - 33 °C is super optimal for normal wheat growth and development. In the present work, heat stress was imposed on plants for 5 h at 40 °C in individual heat stress experiments and in a combined drought/heat stress. Comparatively higher were observed rates of EL in recovered plants after 6th and 7th d of D and DH (Table 2, R6 and R7) in comparison with the same but recovered after 5 d stress (R5). These results correlate with the greater effect of more prolonged D and DH stresses on the wheat cell membrane integrity.

The lowest RWC and highest EL were observed in plants submitted to combined stress and the responses of the two cultivars were quite similar. Analogous observation has been reported by Demirevska *et al.* (2008) in a study on winter wheat response to drought stress.

Protein separation by vertical SDS-PAGE and subsequent characterization by *ImageJ* software indicated that HSPs with low and high Mr, RLS and molecular chaperons HSP70 showed significantly higher translation under D and DH, as compared to the control and H. Immunoblotting analyses proved that RLS, smHSPs and HSP70 contents increased substantially in plants subjected for 7 d to drought, or to DH. Rizhsky *et al.* (2002, 2004) reported similar results for some HSPs (including HSP70 and smHSP families) in *Arabidopsis* and tobacco. The identified basic functions of HSPs mentioned in the Introduction present a reasonable explanation of the observed results. Unlike the above-mentioned proteins, HSP60 chaperone system was slightly affected during combined DH stress (Fig. 1*B*). This is probably due to its major role in protein assembly, *e.g.* Rubisco assembly, and it has been reported that photosynthesis under DH was strongly obstructed (Rizhsky *et al.* 2002, 2004). Increased HSP60 contents (Fig. 1*B*) in recovered leaves support the hypothesis of its chaperoning function during the recovery period, when plants struggle to survive. During this process, maintenance of the Rubisco protein content requires higher content of such chaperones. Apparently, wheat seedlings under such stress conditions preserve stable Rubisco protein contents and occasionally contain even higher RLS quantity. A similar trend of increased RLS under drought has been observed earlier by Demirevska *et al.* (2008). The results reported here demonstrate that this phenomenon occurs also in plants subjected to combined DH stress. Demirevska *et al.* (2008) assumed that a higher Rubisco content could be related to the quick restoration of the protein function during recovery. Scarcely visible trace reactions of HSP110, which fail to produce an informative scanned image on the nitrocellulose membrane, could be explained by the earlier results reported by Vierling (1991), who has proved that 110 kDa protein has been characterized with

transitory expression during the 1st hour of the imposed heat stress. In the present experiment, leaf material from all studied variants was harvested simultaneously, 5 h after the beginning of the heat stress.

As obvious from the SDS-PAGE (Fig. 1*B*), HSPs with low Mr reached the highest contents in D and DH samples, which supports their chaperoning activity (Mansfield *et al.* 1987, Waters *et al.* 1996, Smykal *et al.* 2000). These proteins revealed a specific band about 30 kDa in D. Experimental data also showed that smHSPs were highly represented in control plants. Their accumulation could be provoked either by adverse environmental conditions, or by developmental factors (Mansfield *et al.* 1987, Vierling 1991, Waters *et al.* 1996, Smykal *et al.* 2000). This probably could explain the increased contents of these proteins in the leaves of the experimental plants at an early vegetation stage (8 - 14 d after germination). The high expression of the HSPs with high Mr (above 100 kDa) during D and especially during DH (Fig. 1*A*) confirmed its important role for survival under stress conditions.

SDS-PAGE revealed some expression profile differences between the two tested cultivars. The drought-tolerant Katya exhibited higher RLS, HSP70 and smHSP content, as compared to the sensitive cv. Sadovo during D and DH (Fig. 1*A*).

An increased HSP60 and HSP70 expression was measured in the recovered plants, due to processes related to the repair of protein structure and folding after stress.

In conclusion, the highest HSP expression was established under the combined drought and heat stress in wheat plants. The resulting HSP profile changes resembled the alterations in protein expression provoked by drought stress applied separately. The results differed strongly under individually applied heat shock. Therefore, a simple extrapolation of the results obtained after application of one of the stresses (heat or drought) separately will not produce a reliable basis to predict the effects of their combination. Such opinion was expressed earlier concerning *Arabidopsis* and tobacco by Ron Mittler (2006), who claimed that simultaneous exposure to different abiotic stresses would result in co-activation of the various stress response pathways with synergistic or antagonistic effect and that their combination should be regarded as a new state of abiotic stress in plants.

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