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Effects of heat shock and salinity on barley growth and stress-related gene transcription

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Abstract

The effects of a short (30 min) heat shock (HS) on plants subsequently grown under a salinity stress (SS, 200 mM NaCl) for 10 d were investigated in barley (*Hordeum vulgare* L.) cv. Tokak 157/37. The maximum temperature for HS allowing plant survival was 45 °C. The root length was significantly decreased by SS, whereas HS alone did not affect root growth. Interestingly, HS stimulated root elongation under SS. An osmotic adjustment was promoted in leaves by SS. On the contrary, HS increased the osmotic potential in leaves in the absence of SS, and partly counteracted the effect of SS in the HS+SS treatment. *Cu/Zn-SOD, HvAPX*, *HvCAT2, HSP17, HSP18*, and *HSP90* were transcribed in leaves of HS-treated plants, but not in control plants. The *HSP70* was constitutively transcribed in both the SS and control plants, but after HS, a shorter amplicon was also observed. The genes coding antioxidants, *Cu/Zn-SOD, HvCAT2* and *HvAPX*, were differentially influenced by SS or HS+SS in the roots and leaves. In the roots, the mRNA content of *BAS1, HvDRF1, HvMT2,* and *HvNHX1* increased after the HS treatment. In a recovery experiment in which plants were grown to maturity after HS and HS+SS stress exposure, the plant height increased and the time to maturity was reduced in comparison with SS. Our results show that HS could stimulate plant growth and reduce some of the negative effects of SS, and that it affected the transcription of several stress-related genes.

Additional key words: antioxidants, *Hordeum vulgare*, osmotic adjustment, RT-qPCR, RWC.

Introduction

Salinity is recognized as one of the most important environmental stresses that can severely limit plant growth and productivity (Pitman and Lauchli 2002). High salt concentrations trigger secondary stress factors including osmotic, ionic, and oxidative stresses, resulting in the disruption of ionic homeostasis (Serrano *et al*. 1999, Zhu 2001) and cell membrane integrity by damaging structural and functional proteins (Wang *et al.* 2003). Plant growth is reduced by salinity (Munns and Tester 2008), therefore shoot and root growth rates are useful traits to evaluate plant responses to salt stress (SS) (Munns *et al.* 2000, Munns 2002).

Heat shock (HS) can be defined as exposure of plants to a sudden temperature increase for a short time. Initial effects of high temperature on plants are water loss, growth reduction, alteration of photosynthesis, and oxidative stress (Hasanuzzaman *et al.* 2013). Longer periods of high temperature cause wilting, necrosis, and loss of pigmentation in leaves of herbaceous plants, and supress leaf elongation (Scafaro *et al.* 2010).

Cross tolerance is a property of a plant which exposed to a specific stress acquires resistance to a different stress (Bowler and Fluhr 2000), *e.g*., pre-exposure to heat stress induces salinity and freezing tolerance in yeast and salinity tolerance in tobacco cells (Harrington and Alm 1988, Lewis *et al.* 1995). According to Lewis *et al.* (1995), HS-induced salt tolerance can be associated with trehalose accumulation in some strains of *Saccharomyces cerevisiae*. Lafuente *et al.* (1991) showed that exposure to HS (37 °C) significantly reduces ion leakage (60 %) of cucumber seedlings exposed to a chilling stress at 2.5 °C and made them less sensitive to the effects of cold stress.

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; α-TUB - α-tubuline; BAS1 - 2-*cis*-peroxiredoxin 1; CAT - catalase; Cu/Zn-SOD - copper-zinc superoxide dismutase; DRF1 - dehydration responsive factor-1; GST - glutathione-S-transferase; HS - heat shock; MT2 - methallothionein-like protein type 2; NHX1 - Na^+/H+ antiporter 1; SS - salt stress.
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Improved tolerance to UV-B radiation has been reported in melon seedlings after HS at 45 °C for 1 h (Borisova *et al.* 2001). Conversely, drought stress induces heat stress tolerance by increased membrane thermostability in *Geranium* in association with HSPs and dehydrin accumulation in leaves (Arora *et al.* 1998).

It has been shown that some plants, *e.g.*, tobacco (Harrington and Alm 1988), redbud (Griffin *et al.* 2004), sunflower (Senthil-Kumar *et al.* 2003), and *Salvia splendens* (Natarajan and Kuehny 2008), have a better tolerance to high temperatures or drought after a brief exposure to sublethal high temperatures. Cross tolerance to different chemicals has also been documented (for example, Fedina *et al.* 2009, Ma *et al.* 2013).

Stress-related gene transcription is closely related to plant tolerance mechanisms. Plant superoxide dismutases (SODs), peroxidases (POXs), catalases (CATs), ascorbate peroxidase (APX), and glutathione reductase (GR) are key enzymes reducing the effects of reactive oxygen species (ROS; Gechev and Hille 2005, Miller *et al.* 2010, Petrov and Breusegem 2012). Glutathione-S-transferases (GSTs) are also involved in the protection of tissues against oxidative damages, and their transcription is induced by several factors including pathogens (Chen *et al.* 1996). The *GSTs* have been found to be abundant and constitutively transcribed in drought tolerant barley (Guo *et al.* 2009). Genes belonging to *MYC*, *MYB*, *bZIP*, *EREBP*, and Ap2/ERF transcription factor families are also induced during abiotic stresses in plants (Shinozaki and Yamaguchi-Shinozaki 2000, Mizoi *et al.* 2012).

In barley, expression of several genes has been associated with stress. An *Ap2/ERF* gene and *dehydration responsive factor 1* (*HvDRF1*) has been shown to be involved in the regulation of stress genes by an ABA-dependent pathway (Xue and Loverridge 2004). A *metallothionein-like protein type 2* (*HvMT2*) has been reported to be highly transcribed in barley leaves and roots after exposure to 150 mM NaCl (Öztürk *et al.* 2002). The *NHX1* gene coding for Na^+/H^+ antiport at the tonoplast plays an important role in maintaining K^+ homeostasis by extruding Na^+ from the cytosol into the vacuole during salt stress (Roslyakova *et al.* 2011, Bassil *et al.* 2012).

Although the effects of salinity on barley have been investigated in several studies, it is not known whether a HS pre-treatment affects salinity tolerance. The objective of this study was to investigate the effects of a short HS exposure on the vegetative growth, some physiological parameters, and gene expression of barley seedlings under SS and during recovery.

Materials and methods

Plants and stress applications: Barley (*Hordeum vulgare* L. cv. Tokak157/37) seeds were vernalized at 4 °C for 3 d and planted in 13 cm diameter pots containing *Perlite* (30 g per pot) and kept in a growth chamber (*Angelantoni EHC*, Massa Martana, PG, Italy) at standard growing conditions (a temperature of 25 °C, a relative humidity of 40 %, a 16-h photoperiod, and an irradiance of 600 µmol m^{-2} s^{-1}). The pots were watered by a half-strength Hoagland's solution at two-day intervals. For a preliminary HS treatment, 3-leaf-stage plants were incubated at 25 (control), 35, 45, 50, and 55 °C for 30 min in separate chambers, and then immediately returned to the standard conditions.

The SS treatment was also applied to seedlings at the 3-leaf-stage by transferring them to hydroponic 5-dm^3 containers with half-strength Hoagland's solutions with or without 200 mM NaCl. The nutrient solution was renewed every 3 - 4 d. The SS was applied for 10 d. For HS+SS treatment, HS was carried out at 45 °C for 30 min, then the plants were taken out from the pots, cleaned from *Perlite*, and immediately moved to the hydroponic containers with or without 200 mM NaCl as described above. Temperature in pots was controlled with a thermometer. For each treatment (control, SS, and HS+SS), 3 containers, each with 12 plants, were used.

For recovery experiment, 12 seedlings at 3-leaf-stage were exposed to 200 mM NaCl for 1 h as described. Another group of 12 seedlings were exposed to HS at 45 °C for 30 min before transferring them to SS for 1 h. The seedlings of both groups were then planted in 30-cm diameter pots filled with soil and maintained in a plant growth chamber under above mentioned conditions until flowering. For each experiment, four replicates were used.

Assessment of plant growth and physiological parameters: The measurements of the first, second, and third leaf, and shoots and roots lengths were performed daily for 10 days. Growth rate was then calculated as the average growth per day (mm day^{-1}). Plants were taken out from containers and immediately returned back. In the plant recovery experiment, plant heights were measured at different time points (7, 15, 60, 90, and 150 d after transfer to soil) as the distance from soil surface to the tip of the longest leaf. Days to heading were measured as the number of days from planting until 1/4 of the plants showed inflorescence emergence in each stress group. Spike length excluding awns was measured soon after flowering.

For the measurement of leaf relative water content (RWC), fully expanded leaves (5 - 7 cm) of six plants from each container were excised and fresh mass (FM) was recorded. The leaves were then transferred to Petri dishes containing distilled water and incubated at 4 °C in the dark for 24 h to detect mass of fully water saturated leaves (TM). Dry mass was recorded after drying at 80 °C for 24 h. RWC values were calculated according to a formula described by Barrs and Weatherley (1962): RWC [%] = [(FM - DM)/(TM - DM)] × 100. Using the same procedure, RWC of roots was calculated.

For the measurement of osmotic potential of leaf sap, two leaf pieces (2 - 3 cm) were collected from the second and third leaves of each plant at the 4th day of the treatments. Samples were stored at -20 °C until analysis was performed. The cell sap was obtained by centrifuging leaf segments at 15 000 *g* and 4 °C for 30 min. The osmotic potential was measured by a semi-micro osmometer (*Knauer K-7400*, Germany). Osmometer data were converted to megapascals using a formula [MPa = -c (mosmol kg^{-1}) × 2.58 × 10^{-3}] according to Bagatta *et al*. (2008).

Ion leakage was measured as follows: five 1 cm long pieces were excised from one expanded leaf of nine plants, placed in a glass tube, and washed with distilled water three times. Then 3 cm^3 of distilled water was added to each tube; the tubes were capped and placed in a thermostatically controlled chamber maintained at 25 °C in the dark. After 16 h, the samples (0.06 cm^3) were used for electric conductivity measurement (E1) by a conductivity meter *HORIBA B-137* (Kyoto, Japan). The tubes were then placed in an autoclave (121 °C) for 15 min, and after cooling, conductivity was measured again (E2). Percentage injury was calculated as E1/E2 × 100.

RT-PCR analyses: RNA was extracted from leaf and root tissues. Two biological replicates of two pooled plants were sampled per treatment at each of five time points (0, 0.5, 1, 3, 5 h). Zero hours refers to plants just prior to stress application; the 0.5 h samples were taken 0.5 h after HS and just before the initiation of SS. Five centimeters of leaf pieces from three leaves or 0.5 g of roots collected from two plants were harvested and frozen with liquid nitrogen. Total RNA was purified from tissues by using a *Trizol*® reagent (*Invitrogen,* Grand Island, NY, USA) as recommended by the manufacturer, followed by a *DNase I* (*Fermentas,* Vilnius, Lithuania) treatment in a final volume of 0.1 cm^3. The RNA quality was assessed by agarose gel electrophoresis and the amount of RNA was quantified by using a *NanoDrop* spectrophoto meter (*Thermo Scientific*, Wilmington, DE, USA).

Primer pairs (Table 1 Suppl.) were designed using the *NCBI Primer-BLAST* programme (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and RT-PCR was performed in a 0.050 cm^3 volume with 25 mM $MgCl_2$, 10 mM dNTP, 0.8 U of RNase inhibitor, 5 U of *AMV RTase XL*, 5 U of *AMV-Optimized Taq* DNA polymerase and 20 mM each of forward and reverse primers in a 1× *One Step* RNA PCR buffer (*AccessQuick RT-PCR System*, Promega, Madison, USA). The PCR cycling conditions were as follows: 50 °C for 30 min for reverse transcription and 94 °C for 2 min for inactivation of RNase; then 30 cycles at 94 °C for 30 s, annealing temperatures as in Table 1 Suppl. for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. The amplified samples were then analysed on 1 % agarose gels. Experiments were performed in triplicate.

Real-time quantitative PCR was performed for root samples obtained for the SS and HS+SS treatments at 0, 0.5, 1, 3, and 5 h. Total RNA was extracted and treated with DNAse as described above. First strand cDNA synthesis was performed on 4 µg of total RNA using 100 U of *SuperScriptTM II RT* (*Invitrogen*) and 1 µg of an $Oligo(dT)_{20}$ primer according to the manufacturer's recommendations (a final volume of 0.04 cm^3). All samples from each experiment were reverse transcribed at the same time, and resulting cDNA was diluted 1:4 in nuclease-free water. The RT-qPCR reactions were performed on cDNA samples in a *LightCycler480* (*Roche Diagnostics*, Mannheim, Germany). Amplifications were performed in a total volume of 0.01 cm^3 containing 0.004 cm^3 of cDNA, 1 U of *GoTaq* DNA polymerase (*Promega*), 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.2 µmol of each primer, and 0.001 cm^3 of *EvaGreen* (*Biotium*, Hayward, CA, USA). All primers for RT-qPCR analyses were designed by *Primer-BLAST* using sequences which can be found in the *NCBI* database under accession numbers listed in Table 1 Suppl. The reactions were performed under the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 20 s, annealing temperatures as in Table 1 Suppl. for 20 s, and 72 °C for 20 s followed by a melting curve analysis with a temperature gradient of 0.11 °C s^{-1} from 65 °C to 95 °C. Standard curves were prepared from dilution series of vectors containing cDNAs. Each cDNA was amplified by PCR from control RNA samples and ligated respectively to pTZ57R/T vectors by an *InsTAclone* PCR cloning kit (*Thermo Scientific*). The resulting vectors were used to prepare dilution series with copy numbers arranged as starting from 10^9 to 10^4. In all reactions with the tested genes, the *ACTIN* gene was amplified at the same time using the primers reported in Table 1 Suppl. Raw data were handled with the *LightCycler 480* software (*v. 1.5.0*, *Roche*) and the target gene transcription was normalized using the *ACTIN* transcription for each sample. Two biological replicates were performed.

Statistical analyses: Data were analyzed with the *SPSS v. 21* software (Chicago, IL, USA) using one way analysis of variance (*ANOVA*). Significance of differences between treatment means was analyzed by the Tukey's studentized range (HSD) test. To compare gene transcription values between SS and HS+SS treated plants, the *t*-test was performed in *Excel* assuming homogeneous variances.

Results

To assess the response of barley to HS, the seedlings were exposed at 35, 45, 50, or 55 °C for 30 min, and ion leakage, which is an indication of membrane injury, was assessed. The temperatures higher than 35 °C caused a

significant ion leakage (Fig. 1). Leaf elongation was totally supressed after HS at 50 or 55 °C (Table 1). The elongation of the first leaf was not affected during 10 d of growth after the treatments at 45 °C, whereas the second leaf elongation decreased. The third leaf elongation was significantly higher at 35 °C compared to the control (25 °C) and 45 °C. Finally, the treatment at 45 °C caused leaf tips to turn downwards (Fig. 2*A*). Based on these observations, 45 °C was selected for HS to maximize stress but yet maintaining a slow growth.

Table 1. Effects of heat shock (35 - 55 °C) for 30 min, SS (200 mM NaCl) for 10 d, and HS+SS on leaf, shoot, and root elongations during 10 days. Data are means ± SE (n = 12). Different letters indicate significant differences ($P \leq 0.05$) between treatments according to the Tukey's HSD test.* - no growth was observed.

Treatment	Elongation [mm d^{-1}]		
	first leaf	second leaf	third leaf
Control (25 °C)	3.75 ± 0.08 a	3.28 ± 0.11 a	6.63 ± 0.18 c
HS 35 °C	2.38 ± 0.08 b	2.06 ± 0.12 b	10.14 ± 0.60 a
HS 45 °C	3.54 ± 0.08 a	1.70 ± 0.08 c	8.04 ± 0.80 b
HS 50 °C	*	*	*
HS 55 °C	*	*	*
	shoot	root	
Control	8.50 ± 1.40	3.97 ± 0.96 a	
HS 45 °C	12.20 ± 2.20	3.09 ± 0.54 a	
SS	11.10 ± 6.00	0.70 ± 0.31 c	
HS + SS	*	2.55 ± 0.89 ab	

The interaction of 30 min HS and SS was investigated in this experiment. The shoot elongation rate during 10 d was not significantly affected by the HS or SS treatments alone (Table 1); on the contrary, there was no growth in plants subjected to combined HS+SS. The root elongation was not significantly decreased by HS alone but was dramatically reduced by SS alone. Interestingly, the root elongation under SS+HS was almost normal indicating that HS could induce salt tolerance in the barley roots (Table 1). Root RWC was not affected by the stress treatments, whereas reductions of leaf RWC by HS and HS+SS were significant (Table 2). It appears that HS and not SS was the cause of leaf RWC reduction in this experiment.

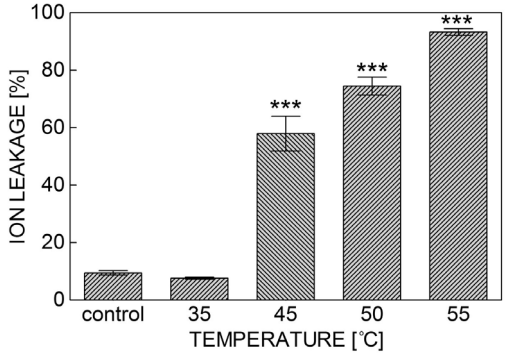

Fig. 1. Ion leakage in barley leaves caused by HS at different temperatures. Means ± SD of three different experiments. *Asterisks* indicate significant differences ($P \leq 0.001$) between the stress groups and control (25 °C) according to the Tukey's HSD test.

Table 2. Effects of stress treatments on leaf RWC, root RWC, leaf osmotic potential, and leaf ion leakage, assessed in plants grown in hydroponics. Measurements were taken immediately before stress application (0 d) and on the fourth day (4 d) (n = 9). The ratios between these two variables were subjected to *ANOVA*, and the Tukey's HSD test was adopted for means separation. Different letters denote significantly different means at $P \leq 0.05$.

Treatments	Leaf RWC [%]			Root RWC [%]			Osmotic potential [MPa]			Ion leakage [%]		
	0 d	4 d	ratio	0 d	4 d	ratio	0 d	4 d	ratio	0 d	4 d	ratio
Control	90.2	89.8	0.99 a	95.6	96.6	1.01	-0.29	-0.34	1.16 c	11.9	8.4	0.74 b
HS	90.2	77.0	0.89 c	94.9	93.4	0.98	-0.24	-0.21	0.89 c	13.5	27.8	2.12 a
SS	88.5	87.1	0.98 a	94.2	92.7	0.98	-0.23	-0.72	3.20 a	11.7	22.0	1.95 a
HS+SS	90.3	81.8	0.90 b	93.3	93.3	1.00	-0.32	-0.64	1.96 b	12.0	30.3	2.55 a

A low leaf osmotic potential was observed in the SS plants but not in the HS plants (Table 2). Surprisingly, HS appeared to counteract partly the effect of SS on osmotic potential in the HS+SS treatment. Membrane injury estimated by ion leakage was significant for all the stress types (Table 2). However, the ion leakage-estimation of membrane injury presented in this work might be affected by various solute concentrations (*i.e.*, driving force) due to different stress applications and thus subjected to a degree of unreliability.

The selected stress-related genes, *HvAPX*, *HvCAT2*, and *HSP90* were transcribed in the leaves of the HS treated plants but not in the control plants (Fig. 2*B*), whereas Cu/Zn-*SOD* was not induced by HS. The HS also induced the transcription of *HSP17* and *HSP18* (Fig. 2*C*). The *HSP70* was constitutively transcribed in both the stressed and control plants, but after HS, a shorter amplicon was observed alongside the expected 608 bp fragment. We did not observe *HSP26* transcription in the control or stressed plants.

The genes coding antioxidants were differentially influenced by SS or HS+SS (Fig. 3). The *Cu/Zn-SOD* transcription was affected by both the stress types. In roots, it was induced transiently by SS after 1 h, but

HS+SS caused a stable induction for 5 h. In leaves, *Cu/Zn-SOD* transcription increased from 1 to 3 h due to SS and then decreased to the pre-stress level at 5 h; HS+SS resulted in an earlier induction (1 h) and a slower decrease of transcription at 3 and 5 h.

The *HvCAT2* was not transcribed in roots exposed to SS only, and a transcript was barely detectable in the HS+SS treatment. On the contrary in leaves, this gene was induced by SS, and HS+SS. The *HvAPX* showed a modulated transcription with SS in roots but a high, stable transcription when HS was also applied. In leaves, its transcription was constant over time, and HS appeared to result in a modest, stable induction. The *HvGST6* seemed to constitutively transcribe in roots, whereas in leaves, the applied stresses resulted in a transient (SS) or stable (SS+HS) transcription increase.

The transcription patterns of four stress-related genes, *HvBAS1, HvNHX1, HvDRF1*, and *HvMT2* under the SS and HS+SS treatments were investigated in roots by RT-qPCR (Fig. 4). Of the four genes studied, the transcriptions of *BAS1* and *HvDRF1* were higher than those of *HvMT2* and *HvNHX1*. The relative transcript amount of *BAS1* was not affected by SS alone but significantly increased immediately after HS and progressively decreased at subsequent sampling times (Fig. 4*A*). The *HvDRF1* transcription was not detectable before the stress (0 h) but was induced in HS exposed roots, and the transcription remained higher than in SS-treated roots until 5 h (Fig. 4*B*). The *HvMT2* was transcribed in both SS and HS+SS roots, and HS+SS resulted in an earlier and higher induction. The *HvMT2* transcription was also significantly different between the stress groups at 1 and 3 h after the treatments (Fig. 4*C*). The *HvNHX1* mRNA was detectable after 5 h only and was more abundant in HS+SS with respect to the SS-treated plants (Fig. 4*D*).

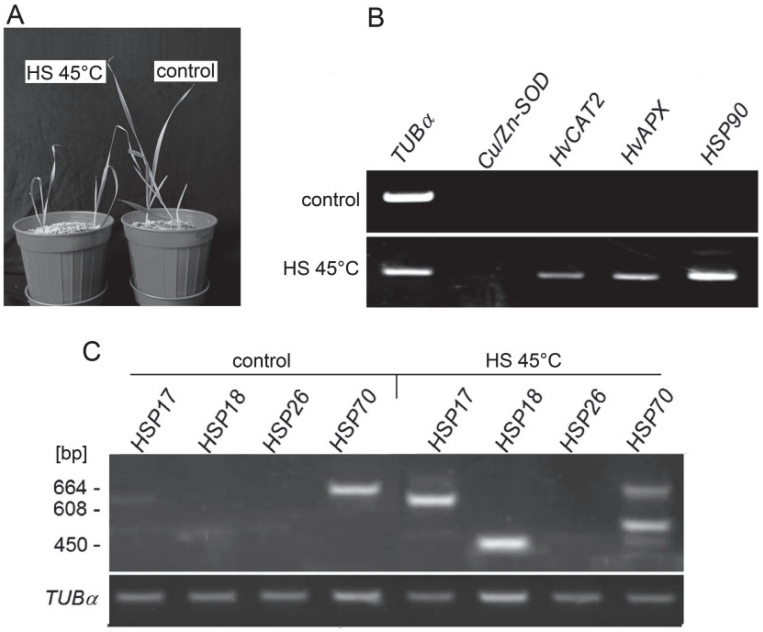

Fig. 2. *A* - Phenotypes of barley seedlings exposed to 45 °C (*right*) and 25 °C (*left*); *B*, *C* - RT-PCR analyses (using primers listed in Table 1 Suppl.) of genes coding antioxidant enzymes (*B*) and heat shock proteins (*C*) in control plants and after HS at 45 °C for 30 min. Barley α-*TUB* was used to normalize gene transcription.

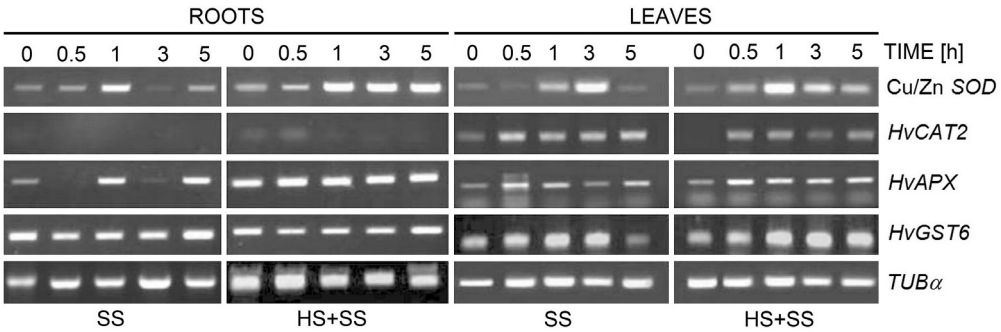

Fig. 3. Transcriptions of four genes coding for antioxidant enzymes in barley seedlings subjected to SS (200 mM NaCl) and HS (45 °C for 30 min) + SS at 0, 0.5, 1, 3, and 5 h after stress application.

In the stress recovery period, the plant height showed a significant increase in the HS+SS and SS-treated plants compared to the controls. In fact, after 150 d of recovery, mean heights were 69.19, 75.33, and 89.6 cm for the control, SS, and HS+SS treated plants, respectively (Fig. 5*A*). The plants subjected to SS or HS+SS developed more rapidly than the controls by elongated internodes (Fig. 5*B*). The HS pre-treatment caused early flowering in the stressed plants. Heading started after 142 d in the HS+SS plants, after 150.8 d in SS plants and

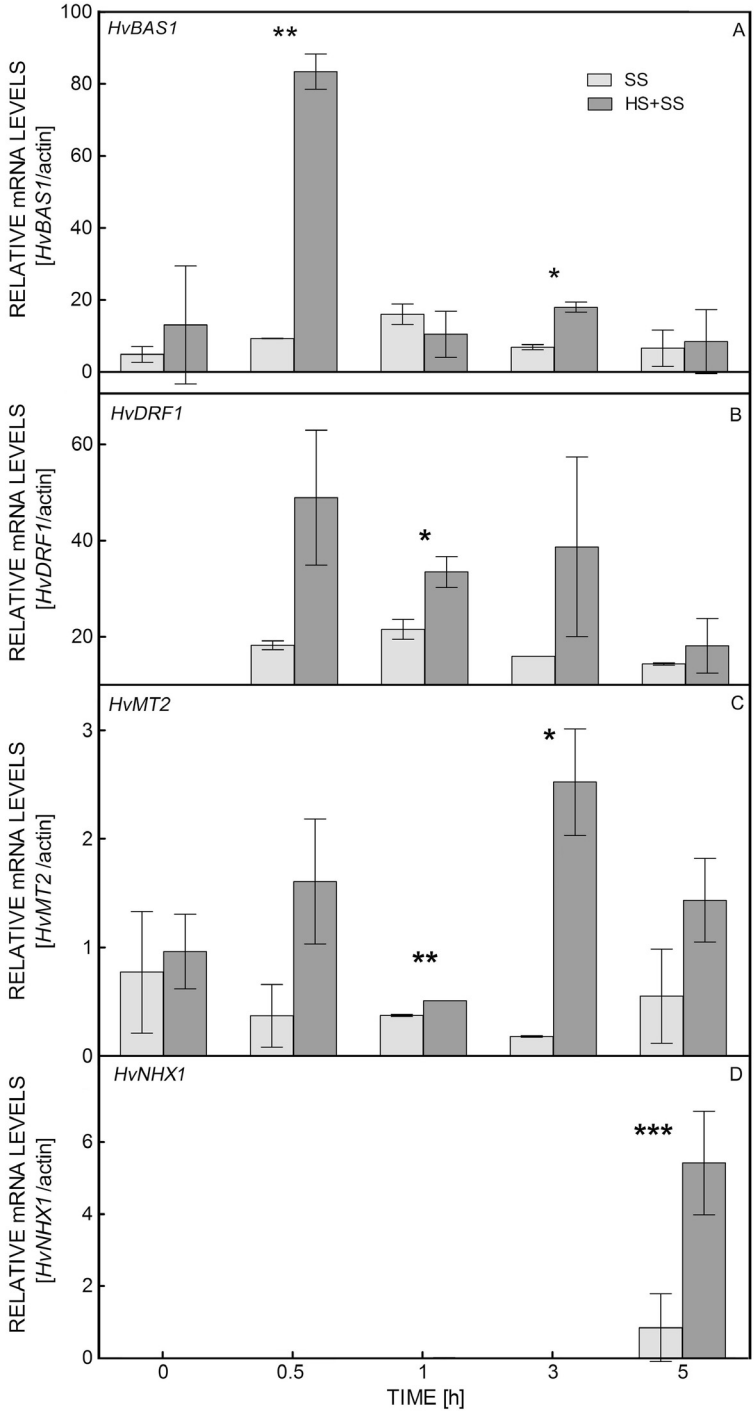

Fig. 4. Relative transcriptions of stress-related genes in barley under SS and HS+SS treatments. Transcriptons were measured by RT-qPCR normalized to *ACTIN*. *Error bars* represent SD of two biological replicates with two technical replicates at each time-point. *Asterisks* indicate significant differences between SS and HS+SS groups at * - $P \leq 0.05$, ** $P \leq 0.01$, and *** - $P \leq 0.001$ according to the *t*-test.

after 147.5 d in the control plants. The spike sizes were strongly and significantly ($P \leq 0.001$) reduced in the SS plants but less in the HS+SS plants with respect to the controls; in the HS+SS plants, the spike length was highly variable, between 4 and 11 cm.

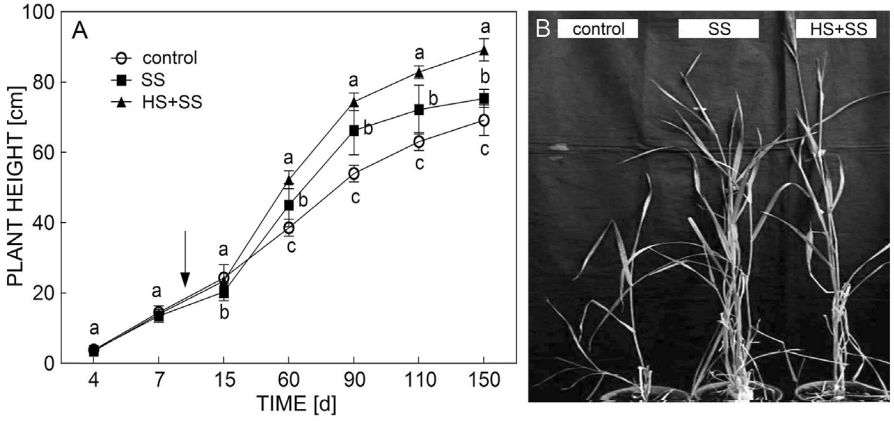

Fig. 5. A recovery experiment. *A* - plant growth during the recovery period. The *arrow* indicates the time of stress application; *B* - an example of stressed and control plants at heading time.

Discussion

The most relevant finding of this work is that HS could stimulate plant growth, which is documented for three traits. Firstly, the elongation of the third leaf significantly increased by HS at 35 °C. Secondly, the strong reduction of root elongation caused by SS was abolished by HS. Thirdly, during recovery from the salt stress, the plant height increased, and the time to maturity was reduced by HS. The fact that the third leaf elongation was enhanced by moderate HS (35 °C) might be due to different resource partitioning between leaves at moderate HS: in fact, increased elongation of the third leaf was paralleled by a decrease of elongation of the first and second leaves.

Leaf growth is affected by salt stress due to changes in transpiration, plasma membrane potential, ABA content, and osmolality in barley (Delane *et al.* 1982, Munns *et al.* 2000, Passioura and Munns 2000, Fricke and Peters 2002, Cramer 2003, Fricke *et al*. 2006). Leaf elongation is inhibited by 40 - 120 mM NaCl (Munns *et al.* 2000, Cramer 2003) and is associated with leaf water status (Passioura and Munns 2000). Under SS, leaf elongation is related to a water potential gradient between leaf xylem and peripheral elongating cells; synthesis of water channel proteins and a plasma membrane potential are also involved in this response (Fricke *et al.* 2006). HS may interact with one or more of these factors resulting in a partial stimulation of growth. It would be interesting to assess whether this is a genotype-specific effect or generally valid for barley.

The attenuation of SS-induced root growth reduction as a consequence of HS indicates that salt tolerance of the root could be triggered by HS with an unknown mechanism. The HS at 45 °C negatively affected leaf RWC after three days, whereas SS alone did not. When the same seedlings were assessed after 16 or 20 d of growth, mean RWC values were further reduced, showing that the short exposure to HS could cause long-term effects on leaf water status. Contrary to our observation, a slight decrease in root RWC in a hydroponic system was observed in salt-stressed barley plants at lower NaCl concentrations (Vysotskaya *et al.* 2010).

Thermotolerance in vegetative tissues has been found to be highly associated with antioxidant capacity, membrane lipid composition, and heat-shock protein synthesis (Maestri *et al.* 2002). Induced transcription of *HSP17, 18,* 26, and *70* by HS were previously shown in barley (Kruse *et al.* 1993). Up to six isoforms of 16.9 kDa HSPs have been detected in a proteomic study (Süle *et al.* 2004). In this work, the perception of HS in the leaves of barley seedlings was confirmed by increased transcriptions of *HSP90, HSP70, HSP17,* and *HSP18.* We consistently obtained a second amplicon, shorter than expected, for *HSP70.* The HSP70 proteins are a large family in plants (Boston *et al.* 1996) and have essential functions in assisting folding *de novo* synthesized polypeptides and translocation precursor proteins in the cell (Wang *et al.* 2004). Incomplete splicing *HSP70* pre-mRNAs because of a high temperature has been shown in maize (Hopf *et al.* 1992), but to our knowledge, the formation of shorter mRNA species has not been reported and encourages further investigation.

Antioxidant enzyme activities change during heat stress showing different profiles depending on stress duration and plant species. A heat stress at 43 °C for 2 h reduces the activities of antioxidant enzymes, such as SOD, CAT, APX, and POX in tobacco leaves (Tan *et al.* 2011). Conversely, SOD, APX, and CAT activities increase in wheat plants grown under a long-term high temperature, whereas GR and POX activities gradually

decrease (Almeselmani *et al.* 2006). The GR and CAT activities are reduced by a heat stress at 46 - 48 °C but increase under 0.4 M NaCl in *Phaseolus* seedlings (Babu and Devraj 2008). The activity of SOD increases, whereas those of APX, CAT, and GR decrease during heat stress in tall fescue and Kentucky bluegrass leaves (Jiang and Huang 2001). In this study, the Cu/Zn-*SOD* transcripts significantly increased 1 and 3 h after the NaCl application and HS+SS in both leaves and roots, whereas HS alone did not induce changes in transcription in leaves. The Cu-Zn SOD is located in both the cytoplasm and nucleus for protection from superoxide anions (Ogawa *et al.* 1996, Alscher *et al.* 2002,). Increase in SOD activity in barley under drought stress has been observed previously (Acar *et al.* 2001). Our study shows that SS induced a rapid Cu/Zn-*SOD* transcript accumulation in both leaves and roots, and that HS likely played a synergistic effect. In cereals, CAT2 is located in the cytosol or peroxisomes of bundle sheath cells (Tsaftaris *et al.* 1983) and its transcript rapidly accumulates after exposure to light (Scandalios *et al.* 1997); as confirmed here, its transcription was not detected in roots. However, we are aware that our transcriptional data are not necessarily correlated with enzyme activities, and therefore they provide only an indication of the involvement of the investigated genes in the HS and SS stress responses.

The BAS 1 is a plastidic 2-Cys type peroxiredoxin which is abundant in plants (Vieira Dos Santos and Rey 2006). Even though peroxiredoxins have fundamental roles in plant tolerance to oxidative stress, the transcription of respective genes under stress is less studied compared to other antioxidant genes. Up-regulation of BAS1 by combined drought and heat stresses in barley has been recently reported (Rollins *et al.* 2013), and *BAS1* transcription during salt stress has been shown in transgenic wheat seedlings overexpressing DREB (Jiang *et al.* 2014). We show that HS caused an immediate increase in *HvBAS1* mRNA content in roots. Similarly, transcription factor *HvDRF1* mRNA was detected only under the stress and was more abundant after HS. The *HvDRF1* is involved in an up-regulation of stress-related gene transcription by an ABA-dependent pathway (Xue and Loverridge 2004). Increase in *HvDRF1* is related to ABA accumulation which was reported 10-fold higher in roots than shoots under salinity (Jia *et al.* 2002). It is not known whether ABA accumulation is enhanced by HS in barley roots.

The MT2 is closely related with metal homeostasis and detoxification, whereas its role in scavenging ROS is not clear (Koh and Kim 2001). In barley, an up-regulation of MT2 was detected by microarray analyses 24 h after NaCl stress (Öztürk *et al.* 2002); consistently, the high HS+SS increased the *HvMT2* transcript accumulation in this work.

Transcription of *HvNHX1* which encodes a vacuolar antiporter, has been previously investigated in barley under SS (Roslyakova *et al.* 2011): an increase in *HvNHX1* transcription and protein content has been demonstrated but not in transcription of *HvNHX2* and *3*. In this work, we observed a similar induction of *HvNHX1* in the SS and HS treated plants in terms of timing; however, the transcription increase was more pronounced in the HS treated seedlings. Although our analyses of gene transcription are limited to the transcriptional level, and no assessment of enzyme activity was performed, they show that the interaction of HS and SS was likely significant in several stress response pathways. Genome-wide transcriptomic and proteomic studies would be needed to have a complete picture of such interaction.

The results from the recovery experiment show that brief exposures to both HS and SS were able to alter phenotypes during subsequent plant development in a complex way: earlier heading was induced by the stress, and HS appeared to counterbalance the negative effects of SS on spike length. The continued effects of brief stress exposure on plant architecture may be associated with complex signalling networks and epigenetic modifications related to plasticity which are not yet well understood (Patel and Franklin 2009, Mirouze and Paszkowski 2011).

Many crop plants are subjected to combined stresses in the field. Assessing their physiological responses and adaptive mechanisms under combined rather than individual stresses may prove to be more realistic and useful. Our results suggest that resolving physiological and molecular responses to combinations of heat and salinity stress can contribute to a better crop management.

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