

Characterization of common and distinctive adjustments of wild barley leaf proteome under drought acclimation, heat stress and their combination

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Abstract In nature, plants are often exposed to combinations of different stresses at the same time, while in many laboratory studies of molecular stress induction phenomena, single stress responses are analyzed. This study aims to identify the common (i.e. more general stress-responsive) and the stress-specific adjustments of the leaf proteome of wild barley to two often co-occurring stress phenomena, i.e. in response to (long-term) drought acclimation (DA) or to (transient) heat stress (HS). In addition, we analyzed those alterations which are specific for the combination of both stresses. Leaf proteome analysis was performed using 2D difference gel electrophoresis followed by protein identification via mass spectrometry with a 1.5 threshold value of changes in relative protein contents. DA resulted in specific upregulation of proteins with cell detoxification functions, water homeostasis maintenance, amino acids synthesis and

lipid metabolism and distinct forms of heat shock proteins (HSPs) and proteins with chaperon functions while proteins related to nitrogen metabolism were downregulated. This response was distinguished from the response to transient HS, which included upregulation of a broad range of HSP products. The common response to both stressors revealed upregulation of additional forms of HSPs and the downregulation of enzymes of the photosynthetic apparatus and chlorophyll binding proteins. The simultaneous exposure to both stress conditions resulted mostly in a combination of both stress responses and to unique abundance changes of proteins with yet unclear functions.

Keywords Detoxification · Difference gel electrophoresis · Drought acclimation · Heat shock proteins · Heat stress · Wild barley

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Abbreviations

BADH	Betaine aldehyde dehydrogenase
DIGE	Difference gel electrophoresis
DA	Drought acclimation
ES	Equilibration solution
HSPs	Heat shock proteins
HS	Heat stress
RWC	Leaf relative water content
MS	Mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
PMF	Peptide mass fingerprint
PSII	Photosystem II
ROS	Reactive oxygen species
RB	Resuspension buffer
RA	Rubisco activase
SHSPs	Small heat shock proteins
2D PAGE	Two dimensional polyacrylamide gel electrophoresis
VWC	Volumetric water content

Introduction

In their natural habitats, plants are exposed to several stresses. These include biological factors like pathogen infections, insect or browsing animals attack, competition by weeds or abiotic stress conditions like water and temperature constraints, salinity, wind, radiation, heavy metal contamination, oxidative stress and others. These stress factors affect plants yield production by more than 50 % (Wang et al. 2003). Drought and high temperature are considered as key stress factors with high potential impact on crop yield. Current climate prediction models indicate a gradual increase in the atmosphere temperature and an increase in the frequency and extent of (transient or long-term) heat stress (HS) periods in the near future for Europe and the Mediterranean (Suzuki et al. 2014). Additionally, high temperatures will be accompanied by extended periods of drought, that could have a negative impact on crop production worldwide and have severe consequences on food security (Rosegrant and Cline 2003; IPCC et al. 2008). Many studies have been conducted to analyze plant response towards one of these stressors (e.g. Fatehi et al. 2012; Ashoub et al. 2013; Budak et al. 2013). However, the combination of two or more stresses like drought and HS is common under field conditions (Shah and Paulsen 2003). It was reported that the responses of tobacco and *Arabidopsis* to a combination of drought and HS were different from the sum of single responses, meaning that one cannot simply extrapolate from the response of plants to drought or HS applied individually to predict the effect of their combination (Rizhsky et al. 2002, 2004). The dynamic molecular changes of plants to survive stress conditions can be studied on the levels of the transcriptome and the proteome. Transcriptome

analyses provide information about gene transcripts alteration levels (Tommasini et al. 2008; Guo et al. 2009). Meanwhile, proteome analyses provide information about changes in protein abundance by modified turnover velocities (Baerenfaller et al. 2012), post translational modification, or involvement of alternative splicing possibilities of transcripts for protein translation. Although being more expensive and difficult than mRNA sequencing techniques, they have the advantage to describe molecular changes directly related to functional alterations of metabolism. Since its discovery (O'Farrell 1975), the two dimensional polyacrylamide gel electrophoresis, 2D PAGE, became a routine approach to analyze proteome changes in response to stresses (Caruso et al. 2009). However, the method is experiencing difficulties in results reproducibility (Bech-Serra et al. 2009). An alternative to the conventional 2D PAGE is the difference gel electrophoresis, DIGE, allowing the sensitive analysis of proteins in a quantitative manner and reducing reproducibility problems. The conventional 2D PAGE or 2D-DIGE followed by mass spectrometry, MS, was successfully used to identify proteome alterations in response several individual stresses including drought in soybean (Alam et al. 2010), heat in wheat (Skylas et al. 2002), salinity in *Arabidopsis thaliana* and rice (Ndimba et al. 2005; Yan et al. 2005), cold in *Arabidopsis thaliana* and rice (Amme et al. 2006; Imin et al. 2004), heavy metals in *Arabidopsis thaliana* (Roth et al. 2006) and others.

Cultivated barley (*Hordeum vulgare* L.) is among the earliest domesticated crops. It ranks fourth in regard to harvested area and tonnage of the world cereal production (<http://faostat.fao.org>). It is cultivated in regions where other cereals cannot be grown due to low rainfall rates, e.g. as a winter crop in the Mediterranean and Minor Asia. Its wild ancestor, *Hordeum spontaneum* (Wild Barley), is mainly distributed in the Mediterranean and Irano-Turanian regions (Harlan and Zohary 1966) as a winter annual species, ranging eastward to Tibet (Choo 2002). Its broad ecological and geographical distribution range supports the accumulation of a high potential for adaptive genetic diversity against various abiotic and biotic stresses beyond what is seen in cultivated barley varieties. It is therefore regarded as a beneficial source for many tolerance genes for breeding programs against stresses (Ellis et al. 2000). This study aims to identify the common and specific changes of wild barley leaf proteome in response to drought, high temperature, and their combination.

Materials and methods

Plant material and stress treatments

Seeds of wild barley from the northern coastal region of Egypt (Wadi Habies 31°24' 0"N/27°03'E) obtained from

Max-Planck Institute for Breeding Research, Cologne, Germany, were used in this study. Seed material was produced from a single plant by two successive selfing propagation cycles. Seeds were germinated on wet filter papers for 2 days. Four seedlings were transplanted into a $16 \times 16 \times 20$ (depth) cm^3 pot filled with 1 kg peat moss medium (Stender AG, Germany) and further regarded as one biological replicate. Seedlings were left to grow in a climate chamber under 16 h photoperiod with light intensity of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperatures of $26 \text{ }^\circ\text{C}$ (light)/ $24 \text{ }^\circ\text{C}$ (dark) and air humidity of 70 %. Soil volumetric water content, VWC, was kept at 30 % using an HH2 moisture meter (Delta-T Devices Ltd, Cambridge, UK). In experimental setup of stress treatments, drought treatment of pot-grown plants was carried out over 3 weeks by withholding water at the stage of two leaves to allow the successive development of an acclimation response, similar to the natural situation. We purposefully did not opt for a treatment with a constant soil water content, since providing water will result in cycles of drought stress and recovery reactions. The soil water potential was measured to be -2.5 MPa (Psypro psychrometer, Wescor, Utah, USA) at the end of drought treatment, prior to sample collection. This long-term drought treatment will be termed “drought acclimation” (DA) (*sensu* Lambers et al. 2008) to distinguish it from short-term (transient) stress treatments, like the applied HS. To simulate HS conditions, which at least somehow resembled a transient, naturally occurring HS, yet not killing the plants, several preliminary experiments had been carried out to identify conditions which are stressful to the plants (as indicated by the occurrence of a K-band in chlorophyll fluorescence measurements, cf. Oukarroum et al. 2007), but which were not lethal (as indicated by full resumption of growth and successful seed formation after the heat treatment). Additionally, HS treatment was applied in delayed time, at the stage of four leaves, to allow samples collection at the same developmental stage of DA treatment. Finally, the following protocol was developed: plants at the stage of four leaves were transferred into a transparent CERTOMAT H hood (Sartorius, Göttingen, Germany) under a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Temperature was increased gradually by one $^\circ\text{C}/20 \text{ min}$ from $26 \text{ }^\circ\text{C}$ until $42 \text{ }^\circ\text{C}$, to allow a limited adaptation of the plants on the level of protein synthesis. Plants were incubated for further 2 h at this temperature. Leaf temperature was checked by an infrared thermometer and air humidity was maintained by supplying water containers to the center of the hood. For combined stresses, plants at the end of DA treatment were subjected to the same heat treatment regime, but omitting the presence of air humidity supplies. The third leaves of three plants/pot were combined for protein extraction, while the corresponding leaf of the fourth plant was used to measure leaf relative water content, RWC, as described by Smart and Bingham (1974). Three biological replicates/treatment were

Table 1 Dye swap arrangement of the differential gel electrophoresis (DIGE) experiment using Cy2, Cy3, and Cy5

Gel number	Cy2	Cy3	Cy5
1	Internal standard	Control 1	Drought 1
2	Internal standard	Control 2	Heat 1
3	Internal standard	Combined stresses 1	Control 3
4	Internal standard	Drought 2	Heat 2
5	Internal standard	Heat 3	Combined stresses 2
6	Internal standard	Drought 3	Combined stresses 3

The experiment includes the Egyptian *H. spontaneum* subjected to drought acclimation, heat stress and their combination compared to the control. The internal standard contained a pool of equal amount of each biological replicate. Three biological replicates were used for each treatment

used for protein extraction and for measuring RWC. Samples were frozen under liquid N_2 , ground with mortar and pestle and stored at $-80 \text{ }^\circ\text{C}$. Samples were collected between 4 and 5 p.m.

Protein extraction, 2D-DIGE and MS analysis

Aliquots of 100 mg ground and frozen tissue were transferred into 2 mL centrifuge tubes and pulverized using mixer mill MM400 (Retsch, Haan, Germany) at a frequency of 25 rpm/s for 30 s. Proteins were extracted using 10 % trichloroacetic acid in acetone containing 10 mM dithiothreitol, DTT, following the protocol of Mèchin et al. (2006). Proteins were resuspended in resuspension buffer (RB: 7 M urea, 2 M thiourea, 30 mM TRIS-HCl and 2 % CHAPS, pH 8.3), sonicated for 3 cycles, 5 s each at 35 % power output using the Sonopuls mini 20 sonicator and MS 1.5 probe (Bandelin, Berlin, Germany). Samples were clarified by two consecutive centrifugation steps at 19,000 g for 15 min each at $20 \text{ }^\circ\text{C}$ and protein concentration was measured (Bradford 1976). An aliquot of 50 μg protein/biological replicate was labeled with fluorescent Cy3, or Cy5 N-hydroxysuccinimide esters following the manufacturer’s recommendations (Lumiprobe, Hannover, Germany) according to the scheme in Table 1. The internal standard consisted of a mixed pool of all biological samples in the experiment at identical protein concentrations, labeled with Cy2. After labeling, volumes were adjusted to 170 μL /reaction mix with RB, an equal volume of $2 \times$ loading buffer (RB containing 2 % DTT, 1 % IPG buffer 4–7) was added, and 18 cm IPG strips 4–7 were hydrated with the sample for the first dimension of the 2 D electrophoresis (overnight at 36 kVh). A parallel strip was rehydrated using 900 μg internal standard to isolate sufficient amounts of proteins for the Matrix-assisted

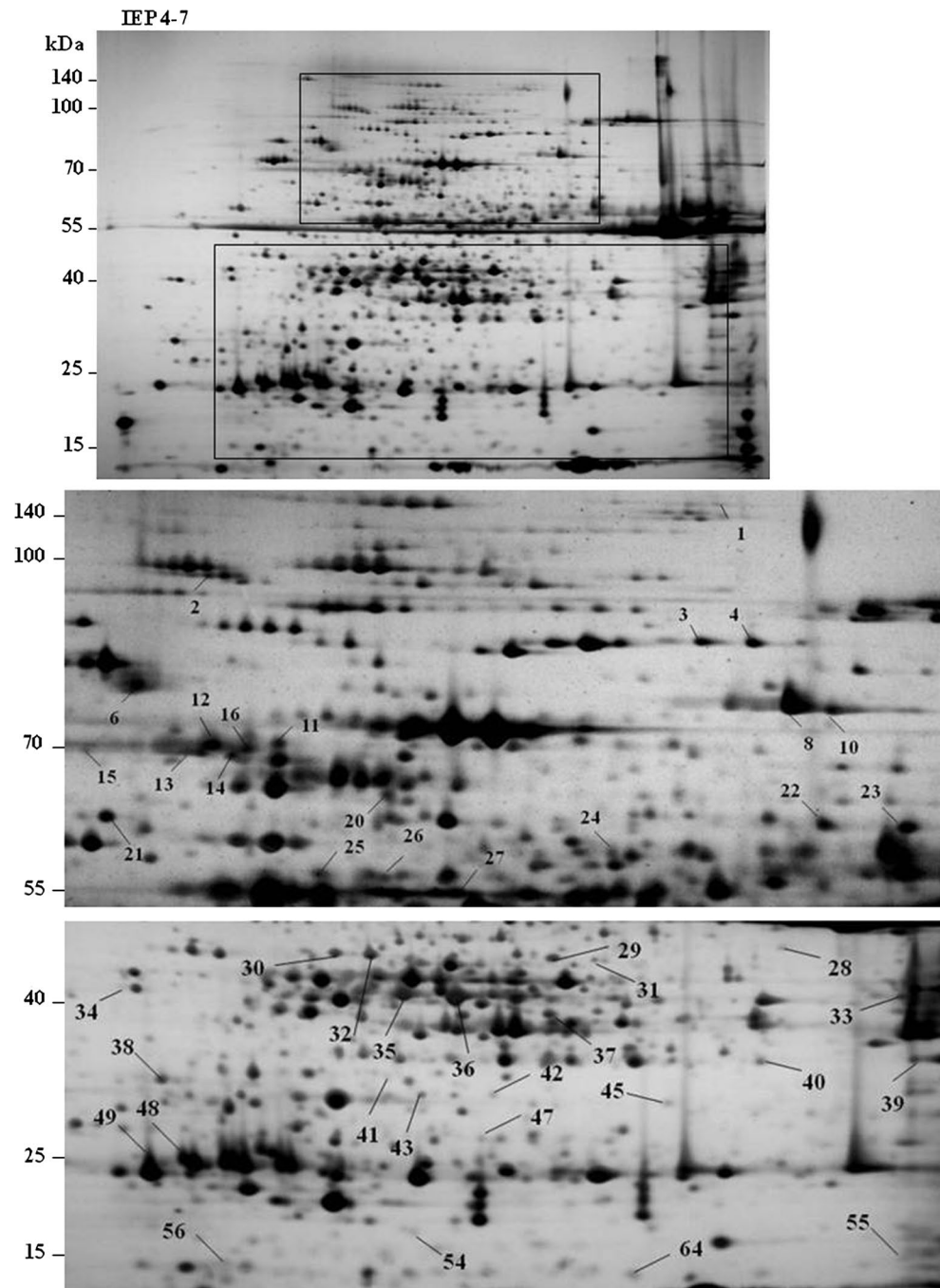
laser desorption/ionization (MALDI) Orbitrap MS analysis. Following electrofocusing and prior to separation on a 18 cm 8–14 % gradient SDS-PAGE (Laemmli 1970), casted into low-fluorescence glass plates, strips were equilibrated for 15 min in 10 mL of SDS equilibration solution [ES: 6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 % (v/v) glycerol, 2 % (w/v) SDS and 0.002 % (w/v) bromophenol blue] containing 1 % DTT, followed by additional 15 min in ES containing 2.5 % (w/v) iodoacetamide. After electrophoresis, gels were scanned using a Typhoon 9410 scanner (GE-Healthcare, Freiburg, Germany) prior to spot visualization by silver staining (Chevallet et al. 2006). One-way ANOVA test and *T* test analysis were carried out on images to distinguish the significance of up- and downregulation of protein spots using DeCyder 2-D Differential Analysis Software (GE Healthcare). The preparative gels with 900 µg internal standard proteins, run in parallel, were stained overnight using colloidal Coomassie blue stain (Neuhoff et al. 1988). Tryptic digestion was carried out on significantly differentially expressed protein spots with at least 1.5-fold threshold changes in relative fluorescence signal intensity and excised from polyacrylamide gels as described by Ashoub et al. (2013) and the included references. Extracted peptides were spotted on a MALDI target using α -cyano-4-hydroxy-cinnamic acid (CHCA, Bruker Daltonics, Bremen, Germany) as MALDI matrix (3 mg/ml in 70 % acetonitrile, 0.1 % trifluoroacetic acid). MS measurements were conducted using two different mass spectrometers to allow identification of the proteins when searching against huge, unreviewed NCBI databases since the barley proteome has neither been fully sequenced nor reviewed. Peptide mass fingerprint (PMF) spectra were acquired with a hybrid LTQ-Orbitrap XL mass spectrometer equipped with a MALDI ion source (Thermo Scientific, Bremen, Germany) due to its high mass accuracy, which is needed for identification via PMF. Twenty-five scans were recorded from each spot with fixed laser energy in positive ion mode between 820 and 4,000 *m/z* with a resolution of 30,000 at 400 *m/z*. For subsequent calibration, a tryptic BSA digest was additionally spotted onto the MALDI target to achieve a mass accuracy of ± 3 ppm. Spectra files obtained from the MALDI-Orbitrap measurements were deisotoped using the vendor's software tool Xtract and saved as MGF files. Peptide mass fingerprint analysis was performed using the Mascot database search engine v2.4 (Matrix Science Ltd.) using the following settings: ± 3 ppm peptide mass tolerance, tryptic enzyme specificity with a maximum of one missed cleavage, carbamidomethylation of cysteine residues as fixed modification and methionine oxidation as variable modification. PMF spectra were searched against the NCBI database restricted to Viridiplantae (green plants). Additionally, MS/MS measurements were performed to confirm the identifications via PMF. For those experiments, a 4800 MALDI TOF/TOF Analyzer (AB Sciex, Darmstadt,

Germany) was chosen due to its superior fragmentation properties (Rietschel et al. 2009). Settings for MALDI-TOF/TOF-MS/MS measurements, spectra processing and data analysis were used as described by Ashoub et al. (2013) (Fig. 1).

Results and discussion

In this study, we used wild barley to identify leaf proteins that are specifically and commonly changed in response to DA and HS, applied alone, and to the simultaneous combination of both stresses. In cereals, most studies were conducted on cultivated plant species due to their commercial importance. However, less is known about the response of their wild relatives to stresses. Studies on (crossable) ancestors may yield invaluable information on available genetic material for improving cultivated cereals. The leaf RWC was used as a parameter to indicate the homogeneity of the water status of the biological replicates prior to protein analysis. RWC of drought and combined stressed plants were reduced to 76 ± 3.9 and 73 ± 1.2 % respectively, in comparison to 96 ± 1.1 % of control plants. In the HS plants, RWC (95 ± 1.6 %) was not significantly changed when compared to control plants. As an alternative to the conventional 2D PAGE, the 2D-DIGE was applied as a quantitative approach to detect protein changes of two samples within a single gel. By application of a specific combination scheme of biological replicates (Table 1), we were able to identify statistically significant differentially expressed spots among treatments. We further introduced the 1.5-fold change threshold of the fluorescence signal of the identified spots between treatments and control to consider protein alterations strong enough prior to be subjected to mass spectrometry (MS) analysis. Protein analysis indicated that out of 1,292 predicted spots by DeCyder 2-D Differential Analysis Software, 103 were statistically differentially expressed in regard to DA, 54 spots in regard to HS and 104 spots in regard to the combination of both stresses. Out of these, 63 proteins underwent an alteration by at least 1.5-fold and were subjected to MS and analysis database search. To confirm protein identification, MS was performed using two different mass spectrometers (cf. Methods section). Peptides mass fingerprint (PMF) and MS/MS fragment spectra were obtained from the same samples. Out of the 63 analysed proteins, only those 45 protein spots that passed the PMF score threshold value and/or were verified by MS/MS are considered in this work. The PMF data for all the identified 63 spots are presented in supplementary Table 1. Proteins identified by MS including their accession numbers, names (where applicable), their up and downregulation levels and their putative functions were categorized to groups in regard to the

Fig. 1 Silver stained image of *H. spontaneum* leaf proteins and a magnification of spot areas (in boxes). First dimension: 18-cm IEFstrips pH 4–7, second dimension: 8–14 % gradient SDS-PAGE. Lines indicate up- or downregulated protein spots subjected to MALDI-MS analysis



applied stress and are summarized in Table 2. Out of the confirmed spots, DA resulted in a specific upregulation of fourteen protein spots while six protein spots were downregulated. HS resulted in a specific upregulation of seven protein spots and downregulation of one protein spot. As a common response to both stresses, four protein spots were upregulated and eight protein spots were downregulated. The unique response of combined stresses (i.e. not found under one stress alone) consisted of the upregulation of one protein spot and downregulation of four protein spots.

Proteome alteration in response to stress conditions

Specific responses to drought acclimation

Under drought stress, stomata are closed to reduce water loss. This results in the decrease of carbon dioxide uptake, reduction of photosynthesis efficiency, an increase in excess excitation energy, and, ultimately, in the accumulation of several ROS and methylglyoxal, which negatively affect proteins, lipids and DNA functions. Under these

Table 2 Proteins from drought and/or heat-treated *Hordeum spontaneum* with ≥ 1.5 -fold alteration compared to control values identified by MALDI mass spectrometry analysis and Mascot database search

SID	Accession number	Protein name/origin	S	C %	M	Mr	PI	D	H	D/H	MS/MS	Function
<i>Drought stress specific proteins alteration</i>												
2	gil475620200	Heat shock 70 kDa protein 4L/Aegilops tauschii	60	17	12	94,042	5.1	1.74		1.27	✓	Protein stability
3	gil326489479	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to lipoxygenase6/ <i>Zea mays</i>	119	21	15	97,142	6.17	2.3		1.7	✓	Lipid metabolism
4	gil326489479	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>Vulgare</i> homologous to lipoxygenase6/ <i>Zea mays</i>	104	22	16	97,142	6.17	3.69		2.11	✓	Lipid metabolism
8	gil50897038	Methionine synthase/ <i>Hordeum vulgare</i> spp. <i>vulgare</i>	108	21	12	84,794	5.68	3.01		2.37	✓	Amino acid synthesis
10	gil326511988	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to mitochondrial NADH-ubiquinone oxidoreductase 75 kDa subunit/ <i>Aegilops tauschii</i>	53	11	7	81,248	6.38	2		1.7	✓	Detoxification
21	gil326532720	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to protein disulfide isomerase/ <i>Triticum aestivum</i>	132	31	15	56,622	4.98	1.5		1.4	✓	Protein stability
22	gil326505210	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to ferredoxin-nitrite reductase precursor/ <i>Triticum aestivum</i>	105	36	17	66,660	6.33	-1.5		-1.8	✓	Nitrogen metabolism
23	gil118430385	ATP synthase CF1 alpha subunit/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i>	118	33	15	55,317	6.32	-1.4		-1.8	✓	Energy/Photosynthesis
24	gil15147871	betaine aldehyde dehydrogenase/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i>	127	35	16	55,276	5.47	1.6		1.5		water homeostasis
28	gil326488463	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to tocopherol cyclase/ <i>Triticum aestivum</i>	62	14	7	52,740	6.87	1.3		1.6	✓	Detoxification
29	gil122220777	S-adenosylmethionine synthase 3/ <i>Hordeum vulgare</i>	132	44	14	43,138	5.51	2.1		2	✓	Amino acid synthesis
30	gil100614	Ribulose-bisphosphate carboxylase activase. large isoform precursor/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i>	221	65	29	47,496	5.68	-1.3		-1.5	✓	Photosynthesis
31	gil122220778	S-adenosylmethionine synthase 2/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i>	156	48	14	43,257	5.58	2.9		2	✓	Amino acid synthesis

Table 2 continued

SID	Accession number	Protein name/origin	S	C %	M	Mr	PI	D	H	D/H	MS/MS	Function
33	gil326507324	predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to mitochondrial formate dehydrogenase/ <i>Aegilops tauschii</i>	88	34	12	41,693	6.51	1.9		1.6	✓	Detoxification
37	gil417356961	glutamine synthetase isoform GS1_2/ <i>Hordeum vulgare</i>	92	33	13	39,035	5.17	-1.5		-1.5	✓	Amino acid synthesis
39	gil326497973	predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i>	88	31	8	39,056	6.27	-1.7			✓	Unknown
40	gil326492141	predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to chloroplastic Ferredoxin-NADP reductase	165	41	17	39,971	8.29	-1.5		-1.4	✓	Amino acid synthesis
41	gil326514324	predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to putative lactoylglutathione lyase/ <i>Aegilops tauschii</i>	90	25	11	38,865	6.88	5.7		2.27		Detoxification
47	gil326507956	predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to glutathione S-transferase/ <i>Pisum sativum</i>	159	52	14	33,484	8.91	3		2	✓	Detoxification
64	gil326517272	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to Actin-depolymerizing factor/ <i>Triticum urartu</i>	151	73	15	16,173	5.93	1.5		1.2		Unknown
<i>Heat stress specific proteins alteration</i>												
6	gil326503698	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to heat shock protein 90/ <i>Triticum aestivum</i>	61	13	8	80,642	4.97		2.31	1.67	✓	Protein stability
11	gil474350516	Chloroplastic ATP-dependent zinc metalloprotease FTSH 1/ <i>Triticum urartu</i>	94	33	14	54,728	5.58		1.53	2	✓	Photosynthesis/ Protein stability
16	gi/326497219	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to heat shock cognate 70 kDa protein/ <i>Triticum urartu</i>	153	32	20	72,202	5.14		2.1	3.8	✓	Protein stability
27	gil326488597	Predicted protein/ <i>Hordeum vulgare</i> ssp. <i>vulgare</i>	123	35	14	52,462	5.93		2	1.9	✓	Unknown
34	gil475622947	chloroplastic Peptidyl-prolyl cis-trans isomerase CYP38/ <i>Aegilops tauschii</i>	55	13	5	44,482	4.7		-1.5	-2.3	✓	Unknown

Table 2 continued

SID	Accession number	Protein name/origin	S	C %	M	Mr	PI	D	H	D/H	MS/MS	Function
42	gil326502706	predicted protein/<i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to chloroplastic 30S ribosomal protein 1/<i>Triticum urartu</i>	74	16	6	32,960	5.79		2.7	1.7	✓	Protein synthesis
45	gil455619	Chloroplast 26 kDa heat shock protein/<i>Hordeum vulgare</i>	84	53	11	26,844	6.03		2.2	6.2		Protein stability
54	gil326519636	predicted protein/<i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to 17.67 kDa heat-shock protein/<i>Helianthus annuus</i>	67	49	7	17,783			4.5	9.8	✓	Protein stability
<i>Common response protein alteration</i>												
12	gil475551788	Heat shock cognate 70 kDa protein/ <i>Aegilops tauschii</i>	185	37	21	71,663	5.09	1.6	1.37	2.1	✓	Protein stability
13	gil326506132	Predicted protein/<i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to heat shock protein 70	163	33	18	71,476	5.07	1.29	1.17	2.2	✓	Protein stability
14	gil762844	Hsc70/ <i>Solanum lycopersicum</i>	109	23	13	71,869	5.08	1.5	1.7	2.7	✓	Protein stability
15	gil567886990	hypothetical protein CICLE_v10033622 mg/ <i>Citrus clementina</i> homologous to heat shock protein 70	88	22	13	71,818	5.17	1.28	1.71	2.15	✓	Protein stability
25	gil110915610	ATP synthase beta subunit/ <i>Catabrosa aquatica</i>	179	64	27	49,429	5.05	-1.7	-1.9	-1.7	✓	Energy/Photosynthesis
26	gil110915610	ATP synthase beta subunit/ <i>Catabrosa aquatica</i>	138	47	18	49,429	5.05	-1.5	-1.5	-1.9	✓	Energy/Photosynthesis
32	gil313574196	ribulose-1,5-bisphosphate carboxylase/oxygenase activase, small isoform/<i>Hordeum vulgare</i> ssp. <i>vulgare</i>	238	74	35	47,292	7.59	-1.3	-1.1	-1.7	✓	Photosynthesis
35	gil313574196	ribulose-1,5-bisphosphate carboxylase/oxygenase activase, small isoform/<i>Hordeum vulgare</i> ssp. <i>vulgare</i>	253	68	37	47,292	7.59	-1.3	-1.2	-1.6	✓	Photosynthesis
36	gil313574196	ribulose-1,5-bisphosphate carboxylase/oxygenase activase, small isoform/<i>Hordeum vulgare</i> ssp. <i>vulgare</i>	222	69	39	47,292	7.59	-1.4	-1.3	-1.7	✓	Photosynthesis
43	gil326502706	predicted protein/<i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to chloroplastic 30S ribosomal protein 1/<i>Triticum urartu</i>	120	27	12	32,960	5.79	-1.7	-1.9	-2	✓	Protein synthesis
48	gil13676406	type I chlorophyll a/b-binding protein b/ <i>Amaranthus tricolor</i>	62	31	7	16,779	4.61	-1.5	-1.6	-1.4	✓	Photosynthesis

Table 2 continued

SID	Accession number	Protein name/origin	S	C %	M	Mr	PI	D	H	D/H	MS/MS	Function
49	gil565405260	Chlorophyll a/b binding protein 1B, chloroplastic-like/ <i>Solanum tuberosum</i>	91	43	8	28,247	5.14	-1.4	-2	-1.2	✓	Photosynthesis
<i>Unique alteration to combined stresses</i>												
1	gil326513540	Predicted protein/<i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to peptidase beta subunit/<i>Zea mays</i>	117	36	15	58,530	6.05			1.53	✓	Unknown
20	gil326512850	Predicted protein/<i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to Bp2A protein, partial/<i>Triticum turgidum</i>	62	38	5	23,969	5.62			-1.5	✓	Unknown
38	gil22607	14-3-3 protein homologue/<i>Hordeum vulgare</i> ssp. <i>Vulgare</i>	92	47	12	29,361	4.83			-1.7	✓	Unknown
55	gil357167288	Chloroplastic-like FKBP-type peptidyl-prolyl cis-trans isomerase 7/ <i>Brachypodium distachyon</i>	141	49	15	28,370	9.61			-1.7	✓	
56	gil326501564	predicted protein/<i>Hordeum vulgare</i> ssp. <i>vulgare</i> homologous to psbP domain-containing protein 3, chloroplastic-like/<i>Brachypodium distachyon</i>	59	52	5	18,558	4.84			-2.7	✓	Unknown

The search was carried out against the entire NCBI nr database restricted to *Viridiplantae* (downloaded Sep 2014, 2,267,064 sequences). SID, spot identification number, S, Mascot MOWSE score, C % percentage of sequence coverage, M number of peptides matched, Mr, calculated molecular weight, pI, isoelectric point of predicted proteins, D drought-induced alteration (fold), H heat-induced alteration (fold), D/H combined stresses-induced alteration (fold), MS/MS peptide sequences additionally confirmed by MS/MS measurements. Only identifications with scores above 76 ($P < 0.05$) and/or identified by additional MS/MS measurements were considered significant. Proteins sequences identified from *Hordeum vulgare* are marked bold, Mr or pI values for proteins from other organisms may deviate from their position in the 2D gel due to sequence variations in the respective proteins from *H. vulgare*

conditions, repair and detoxification mechanisms are activated to prevent damage (Smirnov, 1993). DA treatment resulted in the upregulation of proteins involved in cell detoxification. For instance, the putative lactoylglutathione lyase (#41) glutathione S-transferase (#47) were upregulated. They act as components of the glyoxalase pathway and in antioxidant formation. On the level of lipids protection, tocopherol cyclase (#28) was upregulated. Tocopherols are lipophilic antioxidants and protect against lipid-soluble byproducts of oxidative stress (Smirnov, 1993). In mitochondria the mitochondrial NADH-ubiquinone oxidoreductase (#10) and mitochondrial formate dehydrogenase (#33) were upregulated. The mitochondrial formate dehydrogenase plays a detoxifying role (Li et al., 2002) and 75 kDa mitochondrial NADH-ubiquinone oxidoreductase belongs to the ubiquinone cycle, which plays a role in the plant mitochondrial electron transport chain. It is reduced to ubiquinol which acts as a scavenger of ROS and to the

superoxide radical which is produced during mitochondrial electron transport in plants (Rich and Bonner 1978). On the other hand, during drought stress, plant water content is reduced which requires osmotic adjustment to maintain the metabolic activities of the cells. Betaine aldehyde dehydrogenase (#24) was upregulated during DA. It converts betaine aldehyde to glycine betaine, which mediates osmotic adjustment and is suggested to have positive effects on enzyme and thylakoid membrane integrity and in maintaining photosynthesis efficiency (Ashraf and Foolad 2007). Although its actual role in plant osmotolerance remains to be elucidated, it is thought to play a role in detoxifying free radicals by forming stable complexes with them (Bose et al. 2014).

DA resulted in the upregulation of enzymes which play a role in the biosynthesis of sulfur compounds. Methionine synthase (#8) as well as S-adenosylmethionine synthase 2 and 3 (#29, #31) were upregulated. Methionine synthase

is involved in the synthesis of the amino acid methionine, a key amino acid in protein synthesis and in gene regulation through DNA methylation. It is also involved in the S-adenosylmethionine biosynthesis and regeneration cycle. S-adenosylmethionine synthase is responsible for the formation of S-adenosylmethionine, the methyl donor for transmethylation reactions of proteins, nucleic acids, polysaccharides and fatty acids and the propylamino donor in polyamine biosynthesis. The metabolite is also involved in the control of gene expression, cell proliferation and the production of vitamins important for the development of stress tolerance reactions (Amir 2008). Among proteins involved in lipid metabolism, lipoxygenase 6 (#3, #4) was upregulated. Lipoxygenases are common enzymes in plants, involved in a number of diverse features including growth and development, pest resistance, and senescence or responses to wounding. It was suggested that ethylene, a regulatory signal under drought stress is released by lipid peroxidation through lipoxygenase activity (Smirnov 1993). Grebner et al. (2013) reported that lipoxygenase 6-derived oxylipins are important for the responses to stresses in *Arabidopsis*.

Drought affects the cellular homeostasis and causes protein dysfunction. Therefore, cells utilize efficient mechanisms to allow proteins to maintain their functional conformation and to prevent the aggregation of denatured proteins under stress. During DA, protein disulfide isomerase (#21) was upregulated. It is involved in catalyzing protein folding by formation and breakage of disulfide bonds between cysteine residues within proteins. Heat shock proteins (HSP) 70-4L (#2), with an increased Mr of 94 kDa, was distinctively upregulated. It is distinguished from the cognate HSP 70 species that are constitutively expressed in plants under normal conditions and alter their abundance in response to heat (#16) or combined stress (#12, #14) or from the HSP 70 form which is upregulated as a common response to both stresses (#13, #15). The members of the HSP 70 family play important roles in preventing aggregation and supporting the refolding of non-native proteins under normal and stress conditions (Frydman 2001). They are also involved in organelle protein import and translocation processes and in the proteolytic degradation of unstable proteins by targeting the proteins to lysosomes or proteasomes (Hartl 1996). The overexpression of HSP 70 genes correlates positively with the acquisition of thermotolerance and results in enhanced tolerance to salt, water deficiency and high-temperature stress in plants (Wang et al. 2004). On the other hand, chloroplast proteins related to nitrogen metabolism [ferredoxin-nitrite reductase (#22), chloroplastic ferredoxin-NADP reductase (#40), glutamine synthetase isoform GS1-2 (#37) were downregulated during DA. Ferredoxin-nitrite reductase and glutamine synthetase are involved in nitrogen metabolism. Since plants under

abiotic stress show a deficiency in nitrogen metabolism (Bernard and Habash 2009), we assume that the observed effect of DA was due to a decrease of nitrogen uptake from the soil under drought. In addition, proteins involved in photosynthesis [ATP synthesis: ATP synthase CF1 α (#23) and carbon metabolism: ribulose-bisphosphate carboxylase (Rubisco) activase (RA), large isoform (#30)] were downregulated. Their function is further discussed in the part of combined stress response.

Specific responses to heat stress

Heat negatively affects protein stability and enzyme functions of plants. As a response, plants produce HSPs and proteins with chaperon functions to restore the correct configuration of proteins, to prevent aggregation and to degrade damaged proteins (Wang et al. 2004). In this study HSP 90 (#6), a cognate HSP 70 species (#16), the chloroplast 26 kDa HSP (#45), sHSPs with Mr of 17.6 kDa (#54) and a protein involved in photosynthesis maintenance, chloroplastic ATP-dependent zinc metalloprotease FTSH (#11) were upregulated in response to HS. HSP 90 species have a general role in the refolding of misfolded proteins that can accumulate during plant development or in response to various biotic and abiotic stress conditions, sensing the environment and mediating appropriate phenotypic plasticity (Sangster and Queitsch 2005). sHSPs confer thermotolerance by selectively binding and stabilizing proteins, preventing their aggregation at elevated temperatures, protecting enzymes against heat induced inactivation and facilitating protein refolding by other chaperones (Ganea 2001). Chloroplastic ATP-dependent zinc metalloprotease FTSH 1 is involved in the removal of unassembled or oxidatively damaged D1 proteins from photosystem II, PSII (Adam et al. 2001) and has been suggested to contribute to heat tolerance in grapevine (Rocheta et al. 2014).

In addition, chloroplast 30S ribosomal protein 1 (#42) was upregulated. It is involved in protein synthesis which might reflect the need to produce more proteins with various functions under heat stress conditions.

Common responses to DA and heat stress

Members of the HSP 70 family were upregulated under both treatments. Cognate HSP 70 (#12 and #14), and HSP70 (#13 and #15) were upregulated. On the other hand, proteins involved in photosynthesis were affected as a common response to both DA and HS treatment: rubisco activase (RA) small isoform (# 32, # 35 and #36), ATP synthase β (#25, #26), and type I chlorophyll a/b-binding protein (#48, #49) as well as chloroplast 30S ribosomal protein 1 (#43) were downregulated. In sunflower, photosynthetic assimilation of CO₂ is reduced in response to the

loss of ATP synthase activity under moderate drought and additionally by decreasing Rubisco activity under severe drought stress conditions (Tezara et al. 1999). RA, which regulates Rubisco activity, is sensitive to high temperature. Therefore, inactivation (and degradation) of RA provides a probable biochemical explanation for the inactivation of Rubisco and thus photosynthesis at high temperatures (Feller et al. 1998) as well as under drought stress. Seki et al. (2002) reported the downregulation of 13 *Arabidopsis thaliana* chlorophyll a/b-binding proteins transcripts in response to drought, cold and high salinity conditions. High temperature leads to the dissociation of the inner antenna pigment protein complexes from the central core of the PSII (Georgieva 1999), thus making the chl a/b binding proteins accessible to degradation.

Specific responses to combined stresses

While, in general, heat or drought responsive proteins showed the same behavior under the combined stresses, we also identified alteration of certain proteins, whose response was unique for the combined stresses and included some barley predicted proteins of yet unknown function (Table 2). One protein spot (#1) was upregulated and several proteins with mostly yet unidentified functions (#20, #38, #55, and #56) were downregulated, whose sequences are homologous to predicted proteins in *H. vulgare* or other plant species.

Comparative barley proteome analyses

Proteins detected by barley proteome analyses in other studies in response to stresses differ according to the genotype under study and degree and period of stress. Nevertheless, a comparison with results for *H. vulgare* under drought stress in soil or poly ethylene glycol treatments (Ashoub et al. 2013; Kauser et al. 2013) or under combined drought and heat stresses (Rollins et al. 2013) reveals a common response between both species. For example, methionine synthetase, HSP70 species, glutathione transferase, S-adenosylmethionine synthase, lipoxxygenase, disulfide isomerase, betaine aldehyde dehydrogenase were upregulated in response to drought stress in all cited studies, while RA was downregulated. On the other hand, ATP synthase α subunit was downregulated in response to drought stress or combined drought treatment and HS in *H. spontaneum*, but showed upregulation in one genotype of *H. vulgare*. Several proteins involved in detoxification mechanisms, glyoxylase I, tocopherol cyclase, were not detected in the three cited studies. HSP 70, HSP 90 and ATP-dependent zinc metalloprotease were detected in response to HS by Rollins et al. (2013), but they reported no detected changes of sHSPs. Apart from genetic differences between *H. spontaneum* and

H. vulgare, this difference may be due to the different HS temperatures applied (42 °C in the present study vs. 36 °C in Rollins et al. 2013).

Conclusion

Several studies have been conducted on cultivated barley in response to individual abiotic stress conditions. However, only a limited number of studies have been carried out on combined stresses or on its wild relative. To our knowledge, this is the first study that provides information about the proteome changes of Mediterranean wild barley in response to combined drought and heat stresses. Exposure to a prolonged non-lethal drought stress selectively stimulated the over expression of detoxification enzymes which ameliorate survival under stomatal closure and the subsequent production of reactive oxygen species. Production of HSPs or other proteins with chaperon function was stimulated under both types of stresses. In addition to chaperon species commonly expressed under both stress conditions, stress specific chaperons could be identified. A short time of heat exposure to 42 °C was sufficient to activate the production of a wide range of HSPs as a protection mechanism for other proteins. Both stress conditions showed a negative impact on ATP generation and photosynthesis, as suggested by the downregulation of ATP synthase and Rubisco activase. The combined stresses resulted in unique protein expression alterations which could not be detected by studying each individual stress.

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Conflict of interest The authors declare that they have no conflict of interest.

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